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Targeting Histone Abnormality in Triple-Negative Breast Cancer

PRINCIPAL INVESTIGATOR:

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14. ABSTRACT In this funding period, we continued to test the hypothesis that silencing of key tumor suppressive genes by enhanced crosstalk between LSD1 and HDAC5 is a unique epigenetic mechanism promoting TNBC growth, and blockade of the HDAC5-LSD1 axis results in profound inhibition of TNBC growth and metastasis. By using <i>In vitro</i> pull-down assays with His-tag recombinant LSD1 protein incubating with HDAC5 full-length or deletion mutants and immunofluorescence assays, we identified that HDAC5 domain containing nuclear localization element is essential for interaction with LSD1. To further determine the role of LSD1 in HDAC5 enhanced tumorigenic transformation induced by ICR191, scramble control and LSD1 shRNA lentivirus particles were infected into MCF10A-Vector or MCD10A-HDAC5 cells, which had been treated with ICR191 for 7 months, and the soft agar growth assays have showed that loss of LSD1 in MCF10A-HDAC5 cells significantly abolished cellular ability in colony formation. ChIP studies found that transcriptional de-repression of important LSD1 target genes lies largely in the cooperation between HDAC5 and LSD1 at key active histone marks. Furthermore, we identified that element of -635 to -839 bp as an important regulatory element for HDAC5 transcriptional activity in breast cancer cells. The collaborative studies between initiating and partner PIs have resulted in a publication of research article in <i>Oncogene</i> .					
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1. INTRODUCTION

Triple negative breast cancer (TNBC) is clinically classified based on the absence of the estrogen receptor, the progesterone receptor, or HER2 receptor overexpression. TNBC is more aggressive and distant metastases to lung, liver, brain and bone are more common in TNBC patients than their hormone receptor-positive counterparts. Therefore, new targeted approaches are urgently needed to improve TNBC treatment and prevention. This funded Breast Cancer Breakthrough Award is a partnership between Dr. Yi Huang (initiating PI) and Dr. Nancy E. Davidson (partner PI). In the first funding year, the two PIs worked closely together using multiple *in vitro* and *in vivo* models to investigate the molecular mechanism of crosstalk between HDAC5 and LSD1 and decipher the role of HDAC5-LSD1 axis in promoting transformation of TNBC. We have shown for the first time that LSD1 protein stability is promoted by HDAC5 through the LSD1 associated ubiquitin-proteasome system, confirming that the regulation of LSD1 by HDAC5 is a posttranslational event. In the current reporting period, the two PIs continued to address the mechanism by which crosstalk between HDAC5 and LSD1 leads to the initiation and progression of TNBC, and addressed how HDAC5-LSD1 interaction suppresses expression of tumor suppressor genes (TSGs) to facilitate TNBC development. Our research paper reporting these novel findings was recently published in *Oncogene* (2016 May 23, Epub ahead of print, PMID: 27212032).

2. KEYWORDS

TNBC, HDAC5, LSD1, USP28, sulforaphane, HCI-2509, combination therapy

3. ACCOMPLISHMENTS

a. What were the major goals of the project?

The main goals of this proposal are to: (1) elucidate how changes in interaction of HDAC5 and LSD1 contribute to TNBC initiation and progression; and (2) evaluate the *in vivo* roles of HDAC5-LSD1 axis in TNBC development and test the combinatorial effect of novel inhibitors of HDAC5 and LSD1 in TNBC therapy.

b. What was accomplished under these goals?

Proposed Aims (2 nd year)	Accomplishment
Specific Aim 1: Delineate the molecular basis by which inhibition of LSD1 promotes HDACi-induced apoptosis through reactivation of aberrantly silenced tumor suppressor genes.	The Huang lab investigated the precise mechanisms underlying the physical interaction between HDAC5 and LSD1 in human breast cancer cells. His lab found for the first time that the HDAC5 domain containing nuclear localization sequence (NLS) is essential for interaction with LSD1. His team also examined the effect of HDAC5-LSD1 axis on the expression of LSD1 target TSG in breast cancer cells.
Major Task 1: Determine the role of LSD1/HDACs axis in promoting tumorigenic transformation of TNBC.	Experiments were designed to precisely map the HDAC5 domain(s) responsible for interaction with LSD1. We expressed a series of HDAC5 deletion mutants engineered in

	<p>pcDNA3.1-FLAG plasmids in MDA-MB-231 cells (Figure 1A). Immunoprecipitation assays of cells transfected with full-length HDAC5 complimentary DNA (cDNA) confirmed the HDAC5ΔLSD1 interaction, and deletion of an N-terminal myocyte enhancer factor-2 (MEF2) binding domain (HDAC5- 1) alone had no impact on HDAC5-LSD1 interaction. However, removal of both the MEF2 domain and nuclear localization sequence (NLS) (HDAC5- 2) completely abolished HDAC5ΔLSD1 interaction. Further deletion of an N-terminal HDAC and nuclear export sequence (HDAC5- 3) and MEF2 domain (HDAC5- 4) did not adversely alter LSD1 binding with HDAC5 fragments (Figure 1B). Immunofluorescence studies showed nuclear localization of full-length HDAC5, HDAC5- 1, HDAC5- 3 and HDAC5- 4. Depletion of the NLS-containing domain (HDAC5- 2) completely blocked HDAC5 nuclear translocation (Figure 1C). <i>In vitro</i> pull-down assays by using His-tag recombinant LSD1 protein incubated with HDAC5 full-length or deletion mutants validated that the HDAC5 domain containing the NLS element is essential for interaction with LSD1 (Figure 1D).</p>
<p>Subtask 3: Treat transfected MCF-10A cells for 6 months with mutagen ICR191 and test the potential transformation of transfected MCF-10A cells in 3D culture. (Month 7-15)</p>	<p>In the last reporting period, we demonstrated that overexpression of HDAC5 significantly promoted ICR191-induced colony formation in MCF10A cells. To further determine the role of LSD1 in HDAC5 enhanced tumorigenic transformation induced by ICR191, scramble control and LSD1 shRNA lentivirus particles were infected into MCF10A-Vector or MCD10A-HDAC5 cells, which had been treated with ICR191 for 7 months. Soft agar growth assays showed that loss of LSD1 in MCF10A-HDAC5 cells significantly abolished cellular ability for colony formation (Figure 2).</p>
<p>Major Task 2: Elucidate the role of RGS16 signaling pathway in LSD1-mediated HDACi efficacy in TNBC.</p>	<p>The studies for this subtask are in progress.</p>
<p>Major Task 3: Investigate genome-wide epigenetic gene silencing caused by crosstalk between LSD1 and HDACs.</p>	<p>Please see Dr. Davidson's report.</p>
<p>Other reportable results</p>	<p>1. Effect of HDAC5 on protein acetylation of LSD1/USP28 To examine whether interaction of HDAC5 with the LSD1/USP28 complex deacetylates LSD1 or USP28, <i>in vitro</i> protein acetylation assays was first carried out by incubating GST-tagged recombinant HDAC5 protein with cellular pull-down of LSD1-FLAG or USP28-FLAG by IP, and immunoprecipitates of IgG was incubated with recombinant HDAC5 protein as negative control of assays (Figure 3A). Quantitative immunoblots using antibody against pan-acetylated lysine showed that HDAC5 reduced acetylation level of LSD1 without altering the acetylation status of USP28 (Figures 3B). Next, the <i>in vivo</i> effect of HDAC5 depletion on LSD1 acetylation was investigated in</p>

MDA-MB-231 cells transfected with scramble or HDAC5 siRNAs. After immunoprecipitation with LSD1 antibody or IgG (negative control), immunoblotting was performed and the results showed that expression levels of both total LSD1 protein and acetylated LSD1 protein were decreased by HDAC5 depletion (**Figure 3C**). Acetyl-H3K9 was used as control of substrate and its expression was increased by HDAC5 siRNA (**Figure 3C**). These results suggest that inhibition of HDAC5 alone is not sufficient to increase LSD1 acetylation in breast cancer cells.

2. Inhibition of HDAC5 reactivates expression of LSD1 target genes We next examined whether expression of LSD1 target TSG could be reactivated following HDAC5 inhibition. Loss of expression of cyclin-dependent kinase inhibitor p21 and epithelial marker claudin-7 (CLDN7) has been reported to be associated with an aggressive phenotype of breast cancer. The transcription activity of p21 and CLDN7 has been found to be suppressed by enhanced activity of LSD1 in breast cancer. Transfection of HDAC5 siRNA resulted in significantly increased mRNA expression of p21 and CLDN7 in MDA-MB-231 cells (**Figure 4A**). Quantitative chromatin immunoprecipitation assays revealed that depletion of HDAC5 decreased occupancy of both HDAC5 and LSD1, and increased enrichment of H3K4me2 and acetyl-H3K9 at the promoters of both genes (**Figure 4B**). These data suggest that transcriptional de-repression of these genes lies largely in the cooperation between HDAC5 and LSD1 at key active histone marks.

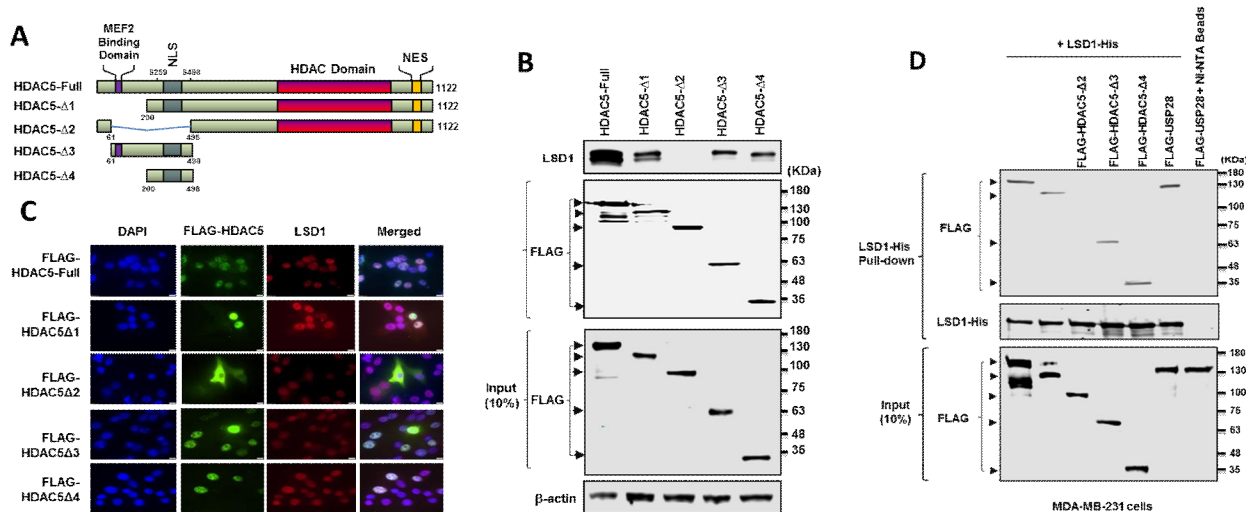


Figure 1. HDAC5 interacts with LSD1 through NLS element . (A) Schematic representation of full length and deletion mutants of HDAC5-Flag constructs. **(B)** FLAG-tagged full-length or deletion mutants of HDAC5 were expressed in MDA-MB-231 cells. Extracts were immunoprecipitated with anti-FLAG antibody, and bound LSD1 was examined by IB using anti-LSD1 antibody. IB with anti-FLAG was used to detect the levels of FLAG-tagged HDAC5 full-length or deletion mutants in IP and input samples (10%). **(C)** MDA-MB-231 cells were transfected with plasmids expressing FLAG-tagged full-length or deletion mutants of HDAC5 proteins. Immunofluorescence study was performed using anti-FLAG antibody. 4,6-Diamidino-2-phenylindole was used as a control for nuclear staining. All the experiments were performed three times with similar results. **(D)** The whole cell lysates of MDA-MB-231 cells expressing full length or truncated mutants of HDAC5-FLAG or full length of USP28-FLAG were incubated with the recombinant His-tagged LSD1 protein. Ni-NTA resin was then added to mixture of cell lysate and His-tagged LSD1 protein. After washing with buffer containing imidazole, the beads were re-suspended in elution buffer containing imidazole and then subjected to immunoblot with anti-FLAG or anti-LSD1 antibodies. The experiments were performed three times with similar results.

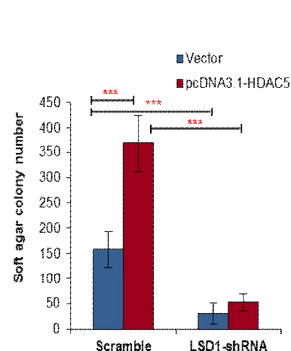


Figure 2. MCF10A cells transfected with pcDNA3.1 or pcDNA3.1-HDAC5 plasmids were treated with dimethyl sulfoxide or 500 ng/ml ICR191 for 7 months followed by soft agar colony formation assays.

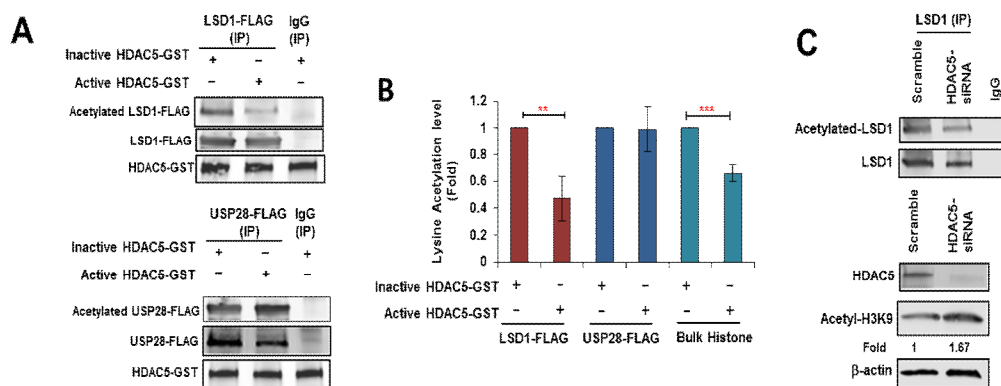


Figure 3. Effect of HDAC5 on protein acetylation of LSD1/USP28 (A) The immunoprecipitates of FLAG using FLAG-M2 agarose from MDA-MB-231 cells overexpressing FLAG-tagged USP28 or FLAG-tagged LSD1 were used as substrates for protein deacetylation assay. IgG was used as negative control. Active or heat inactivated recombinant human GST-tagged HDAC5 protein were mixed with immunoprecipitates and incubated at 37 °C for 6 h as described in ‘Materials and Methods’. The reactions were then subjected to immunoblots with anti-acetyl lysine antibody. FLAG-tagged USP28 or LSD1 proteins were probed with anti-FLAG antibody. HDAC5-GST protein was probed with anti-HDAC5 antibody. (B) Histograms represent the means of levels of acetyl-LSD1, acetyl-USP28 and acetyl-histone determined by quantitative IB using infrared IB detection and analysis. (C) MDA-MB-231 cell transfected with scramble or HDAC5 siRNAs for 48 h. LSD1 or IgG antibodies were added to cell lysate. IP was performed with anti-LSD1 antibody followed by IB with anti-acetyl lysine and anti-LSD1 antibodies, respectively. Effect of HDAC5 siRNA on AcetylH3K9 protein expression in MDA-MB-231 cells was examined by IB with anti-acetyl-H3K9 antibody.

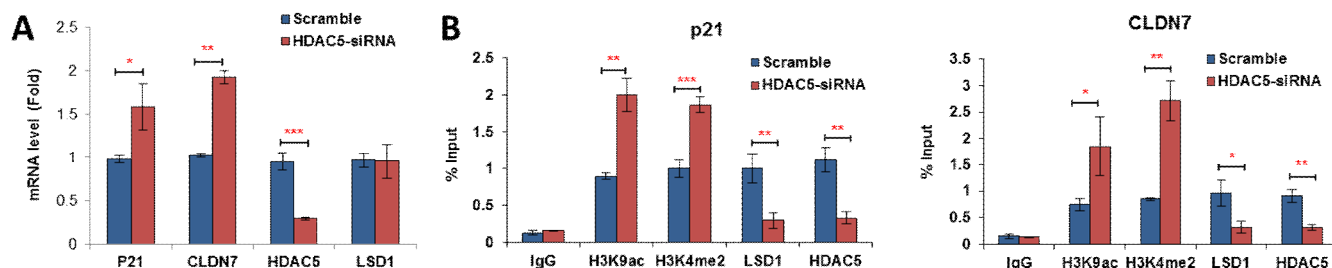


Figure 4. Effect of HDAC5 on transcription of LSD1 target genes. (A) mRNA expression of indicated genes in MDA-MB-231 cells transfected with scramble siRNA or HDAC5 siRNA. Data are means \pm s.d. of three independent experiments. (B) Quantitative chromatin immunoprecipitation (ChIP) analysis was used to determine the occupancy by acetyl-H3K9, H3K4me2, LSD1 and HDAC5 at promoters of p21 or CLDN7 in MDA-MB-231 cells transfected with scramble or HDAC5 siRNA. * P <0.05, ** P <0.01, *** P <0.001, Student’s t -test.

Proposed Aims	Accomplishment
Specific Aim 2: Elucidate the role of LSD1 in HDACi therapy and chemoprevention of TNBC in animal models.	Dr. Huangø team assisted Dr. Davidsonø team in designing and performing the animal studies.

Subtask 3: Evaluate combination strategies using LSD1i and HDACi in different subtypes of breast tumors (Month 10-26).

In the first funding year, our studies showed that a natural HDAC inhibitor, sulforaphane (SFN), destabilizes LSD1 through inhibition of HDAC5 transcriptional activity. To precisely address the mechanism by which SFN down-regulates HDAC5 transcription, successive deletion constructs of HDAC5 5' flanking region luciferase reporter pGL2 vectors were engineered into luciferase reporter pGL2 vectors (**Figure 5A**). The plasmids were transiently transfected into MDA-MB-231 cells followed by quantitative luciferase activity assay. Using full length HDAC5 promoter (P1) as an experimental control, we found that deletion of downstream element +17 to +666 (P2) or additional deletion of upstream element -839 to -1251 (P3) had no significant impact on reporter gene activity. Further deletion of -635 to -839 significantly attenuated the luciferase reported gene activity (**Figure 5B**). The result of these assays suggest that regulatory activity at element of -635 to -893 bp of HDAC5 promoter might play an essential role in mediating transcriptional activity of the HDAC5. These data shed light on the mechanisms underlying transcriptional regulation of HDAC5 promoter activity in breast cancer cells and identify -635 to -839 as an important regulatory element for HDAC5 transcription.

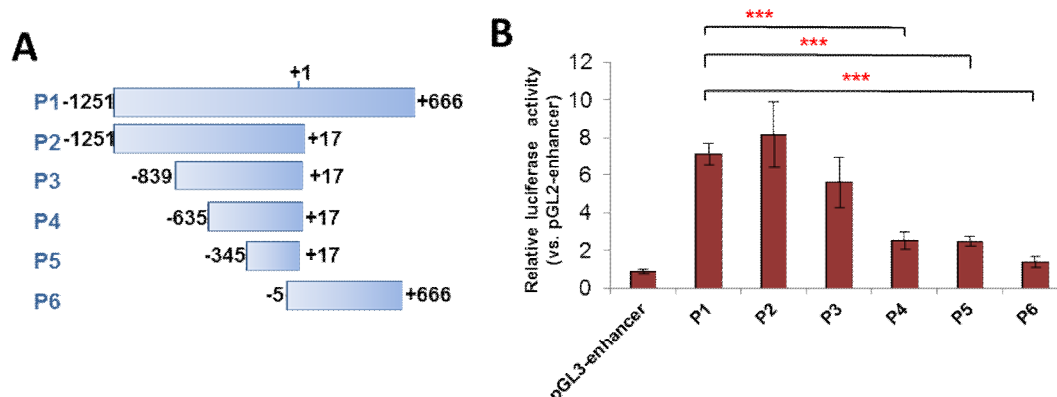


Figure 5. Analysis of HDAC5 promoter activities. (A) Deletion constructs of the HDAC5 flanking region. (B) MDA-MB-231 cells were transfected with pGL2-enhancer or constructs of pGL2-enhancer-HDAC5 promoter elements with co-transfection of pRL-TK. Reporter gene activities were measured after 48 h of transfection. The relative luciferase activity of fragments P2-P6 was compared with that of full length P1. Transfection of pGL2-enhancer was used as negative control. * $p < 0.05$. ** $p < 0.01$, student t-test.

b. What opportunities for training and professional development has the project provided?

This award has provided an excellent vehicle for postdoctoral fellows and students working on this project to advance their career goal in breast cancer and transition to an independent investigator. Dr. Chunyu Cao has completed his postdoc fellow training at UPCI and obtained an independent faculty position at a Chinese university. He will continue to carry out breast cancer

research during his faculty tenure. This award also provided excellent research opportunities for UPCI Academy summer student and visiting student from Tsinghua University, China.

c. How were the results disseminated to communities of interest?

This work has been published in *Oncogene*: Cao C, Vasilatos SN, Bhargava R, Fine J, Oesterreich S, Davidson NE, Huang Y. Functional interaction of histone deacetylase 5 (HDAC5) and lysine-specific demethylase 1 (LSD1) promotes breast cancer progression. *Oncogene*, 2016. PMID: 27212032

UPCI 2016 Retreat abstract: Vasilatos SN, Katz TA, Yatsenko TA, Chen L, Luthra S, Chandran UP, Oesterreich S, Davidson NE, Huang Y. Genome-wide effect of inhibition of lysine-specific demethylase 2 (LSD-2) on gene expression and chromosomal stability in human breast cancer.

What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, we plan to carry out experiments to precisely characterize the interaction domains and structures of the HDAC5-LSD1-USP28 complex and study how the interplay between HDAC5 and USP28 regulates LSD1 protein stability. We will also determine how synchronized gain-of-function of HDAC5 and USP28 facilitates TNBC tumorigenesis in an LSD-dependent manner. The impact of the HDAC5-LSD1 axis on induction of phenotypic characteristics of TNBC cancer stem cell-like cells (CSLCs) will also be investigated. We will expand our exploratory immunohistochemistry study into a larger set of primary and metastatic TNBC patient tumor samples to establish the potential correlation between HDAC5/LSD1 overexpression and advanced clinical stage of TNBC. We will determine how HDAC5 mRNA expression is up-regulated in TNBC cells, and dissect the mechanisms of how suppression of HDAC5 transcription facilitates USP28/LSD1 degradation to inhibit TNBC growth. In addition, the mechanisms of how HDAC5 inhibitors sensitize TNBC cells to novel LSD1 inhibitors will be characterized. The combinatorial impact of novel inhibitors of HDAC5 and LSD1 on growth of PDX TNBC tumors will be assessed to aid in establishing clinical relevance.

4. IMPACT

(a) What was the impact on the development of the principal discipline(s) of the project?

Despite the promising results produced by HDAC inhibitors (HDACi) in treatment of hematological cancers, little clinical evidence exists to indicate that HDACi work effectively against solid tumors including breast tumor as a monotherapy. Thus it is critically important to develop novel therapeutic strategies to improve the efficacy of HDACi and reduce the side effects by targeting more specific regions of chromatin and the subset of genes that are associated with most prominent alterations in the breast cancer genome. We have successfully shown that sulforaphane, a natural bioactive HDAC inhibitor, suppresses HDAC5 expression that in turn destabilizes LSD1 protein. Our preclinical data strongly suggest that targeting HDAC5-LSD1 pathway by sulforaphane in combination with a potent LSD1 inhibitor HCI-2509 may represent an excellent approach for overcoming the nonspecific side effects of HDAC inhibitors in treating patients with triple negative breast cancer. Our work has opened a new avenue for the potential utility of crosstalk between histone demethylation and deacetylation as a new therapeutic target in breast cancer. As epigenetic therapies are currently being clinically tested, this new strategy has great potential to be rapidly translated into clinical trials.

(b) What was the impact on other disciplines? Nothing to Report

(c) **What was the impact on technology transfer?** Nothing to Report

5. CHANGES/PROBLEMS

(a) Changes in approach and reasons for change

No major changes in approach have been made since the initiation of the award.

(b) Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report

(c) Changes that had a significant impact on expenditures

Nothing to Report

(d) Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

6. PRODUCTS

(a) Publications, conference papers, and presentations

Research article:

Cao C, Vasilatos SN, Bhargava R, Fine J, Oesterreich S, Davidson NE, Huang Y. Functional interaction of histone deacetylase 5 (HDAC5) and lysine-specific demethylase 1 (LSD1) promotes breast cancer progression. *Oncogene*, 2016. PMID: 27212032

Meeting abstract and presentation:

Vasilatos SN, Katz TA, Yatsenko TA, Chen L, Luthra S, Chandran UP, Oesterreich S, Davidson NE, Huang Y. Genome-wide effect of inhibition of lysine-specific demethylase 2 (LSD-2) on gene expression and chromosomal stability in human breast cancer. UPCI 2016 Retreat

(b) Website(s) or other Internet site(s) Nothing to Report

(c) Technologies or techniques Nothing to Report

(d) Inventions, patent applications, and/or licenses Nothing to Report

(e) Other Products Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

a) Individuals who have worked on the project

Name:	Yi Huang	Nancy Davidson	Shauna Vasilatos	Chunyu Cao	Ye Qin
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Project Role:	Initiating-PI	Partner-PI	Technician	Postdoc Fellow	Postdoc Fellow
Researcher Identifier (e.g. ORCID ID):	N/A	N/A	N/A	N/A	N/A
Nearest person month worked:	3.6	1.2	9.0	6.0	6.0
Contribution to Project:	Designed and oversaw the studies to define the basic mechanisms and biological consequences of the functional interplay between HDAC5/LSD1 in breast cancer	Oversaw IHC studies and animal experiments, and interpreted the results generated from <i>in vivo</i> studies	Performed IHC and microarray studies	Studied molecular mechanisms by which LSD1 and HDAC interacted, and carried out animal study	New Postdoc
Funding Support:	CDMRP Breast Cancer Breakthrough Award, Breast Cancer Research Foundation	CDMRP Breast Cancer Breakthrough Award	CDMRP Breast Cancer Breakthrough Award	CDMRP Breast Cancer Breakthrough Award	CDMRP Breast Cancer Breakthrough Award

b) Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Dr. Chunyu Cao has completed his postdoctoral fellow training, and Dr. Ye Qin joined the laboratory to carry out the work as a postdoctoral fellow.

c) What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Partnering PI, Dr. Nancy E. Davidson, will submit her annual report separately.

QUAD CHARTS: N/A

9. APPENDICES: updated curriculum vitae is attached

CURRICULUM VITAE

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School of Medicine

BIOGRAPHICAL

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EDUCATION and TRAINING

Date Attended	Name and Location of Institution	Degree Received and Year	Major Subject
Undergraduate 1986-1991	Nanjing Medical University, Nanjing, Jiangsu, P.R. China	M.D., 1991	Clinical Medicine
Graduate 1996-2001	Medical University of South Carolina, Charleston, SC, USA	Ph.D., 2001	Pathology and Lab Medicine
Postgraduate 1991-1994	Affiliated Hospital of Medical College of Nanjing University, Nanjing, Jiangsu, China	Residency	Surgery
2001-2005	Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD, USA	Postdoctoral fellow	Oncology

APPOINTMENTS and POSITIONS**ACADEMIC (Institutional):**

Years Inclusive	Name and Location of Institution or Organization	Rank/Title
2006-2009	Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD	Research Associate (Faculty)
2009-2015	Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA	Research Assistant Professor
2010-	Cancer Therapeutics Program (2010-2012), Womens Cancer Research Center (2012-), Breast and Ovarian Cancer Program (2014-), University of Pittsburgh Cancer Institute, Pittsburgh, PA	Member
2015-	Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA	Assistant Professor

MEMBERSHIP in PROFESSIONAL and SCIENTIFIC SOCIETIES

Organization	Year
The American Association for Cancer Research (Active Member).	1998-
The America Association for Advancement of Science	1999-2001, 2013-2015

HONORS

Honors and Awards	Year
1 st Prize of 32 nd Annual Research Day, Medical University of South Carolina.	1997
Young scholar award for the 8 th International Symposium of SCBA in Hong Kong.	1999
DOD breast Cancer Postdoctoral Fellowship Award	2002
Hodson Young Investigator in Oncology, Johns Hopkins Sidney Kimmel Comprehensive Cancer Center.	2007
Invited lecturer of Gordon Research Conference on Polyamines	2009
Samuel and Winters Foundation award for Medical Research	2011
Competitive Medical Research Fund (UPMC) Award	2012
Director's award of basic science at UPCI annual retreat poster competition (senior author)	2013
DOD Breast Cancer Breakthrough Award	2014

PUBLICATIONS

I. Research Articles

1. **Huang Y**, Johnson KR, Norris JS, Fan W. NF- κ B/I κ B signaling pathway may contribute to the mediation of paclitaxel-induced apoptosis in solid tumor cells. Cancer Res., 60: 4426-4432, 2000. PMID: 10969788
2. **Huang Y** and Fan W. I κ B Kinase activation is involved in the regulation of paclitaxel-induced apoptosis in human tumor cell lines. Mol. Pharmacol., 61: 105-113, 2002. PMID: 11752211
3. **Huang Y**, Fang Y, Dziadyk JM, Norris JS, Fan W. The possible correlation between activation of NF- κ B/I κ B pathway with the susceptibility of tumor cells to paclitaxel-induced apoptosis. Oncology Res., 13: 113-122, 2002. PMID: 12392159
4. **Huang Y**, Hager ER, Phillips DL, Dunn VR, Hacker A, Frydman B, Kink JA, Valasinas AL, Reddy VK, Marton LJ, Casero RA, Davidson NE. A novel polyamine analog inhibits growth and induces apoptosis in human breast cancer cells. Clin. Cancer. Res., 9: 2769-2777, 2003. PMID: 12855657 PMCID: PMC3625930
5. **Huang Y**, Fang Y, Wu J, Dziadyk JM, Zhu X, Sui M, Fan W. Regulation of vinca alkaloid-induced apoptosis by NF- κ B/I κ B pathway in human tumor cells. Mol. Cancer Ther. 3: 271-277, 2004. PMID: 15026547
6. **Huang Y**, Keen JC, Hager ER, Smith R, Frydman, B, Valasinas AL, Reddy VK, Marton LJ, Casero RA, Davidson NE. Regulation of polyamine analogue cytotoxicity by c-Jun in human cancer MDA-MB-435 Cells. Mol. Cancer Res., 2: 81-88, 2004. PMID: 14985464
7. **Huang Y**, Pledge A, Rubin E, Marton LJ, Woster PM, Sukumar S, Casero RA, Davidson NE. Role of p53/p21^{WAF1/CIP1} activation in the mediation of polyamine analogue induced growth inhibition and cell death in human breast cancer cells. Cancer Biol. Ther., 4(9):1006-1013, 2005. PMID: 16131835 PMCID: PMC3639297
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9. **Huang Y**, Keen JC, Pledge A, Marton LJ, Zhu T, Sukumar S, Park BH, Blair BG, Brenner K, Casero RA, Davidson NE. Polyamine analogues down-regulate estrogen receptor α expression in human breast cancer cells. J. Biol. Chem., 281(28): 19055-19063, 2006. PMID: 16679312 PMCID: PMC3623667
10. Abukhdeir AM, Blair BG, Brenner K, Karakas B, Konishi H, Lim J, Sahasranaman V, **Huang Y**, Keen JC, Davidson NE, Vitolo M, Bachman KE, Park BH. Physiologic estrogen receptor alpha signaling in non-tumorigenic human mammary epithelial cells. Breast Cancer Res. Treat., 99(1):23-33, 2006. PMID: 16541319
11. Babbar N, Hacker A, **Huang Y**, Casero RA. Tumor necrosis factor α induced spermidine/spermine N¹-Acetyltransferase (SSAT) through Nuclear Factor κ B in non small cell Lung cancer cells. J. Biol. Chem., 281(34): 24182-24192, 2006. PMID: 16757480
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II. Review Articles

1. Fan W, Sui M, **Huang Y**. Glucocorticoids selectively inhibit paclitaxel-induced apoptosis: mechanisms and its clinical impact. Curr. Med. Chem., 11: 403-411, 2004. PMID: 14965221
2. **Huang Y**, Pledge AM, Casero RA, Davidson NE. Molecular mechanisms of polyamine analogues in cancer cells. Anti-Cancer Drugs, 16(3): 229-241, 2005. PMID: 15711175
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4. Katz TA, **Huang Y**, Davidson NE, Jankowitz JC. Epigenetic reprogramming in breast cancer: from new targets to new therapies. Annals of Medicine, 46(6):397-408, 2014. PMID:25058177

III. Book Chapters

1. **Huang Y** and Davidson NE. Chapter 74: Breast cancer. In: Principles of Molecular Medicine (2nd ed). Runge, M., and Patterson, WC. (eds), Humana Press, p728-735, 2006.
2. **Huang Y**, Woster PM, Marton LJ, Casero RA, Polyamine analogues targeting epigenetic gene regulation. Essays Biochem, Portland press, 46:95-110, 2009. PMID: 20095972 PMCID: PMC3564236
3. **Huang Y**, Shaw PW, Davidson NE. Chapter 22: Inhibition of histone deacetylation. In: Epigenetics Protocols II, Tollefsbol, TO (ed.), Methods in Molecular Biology, vol. 791, Springer Science, 791:297-311, 2011. PMID:21913088
4. **Huang Y**, Woster PM, Marton LJ. Chapter 10: The design and development of polyamine-based analogues with epigenetic targets. In: Polyamine Drug Discovery, Woster, PM., and Casero, RA. (eds). Royal Society of Chemistry Drug Discovery Series No. 17, Thomas Graham House, p238-256, 2012.

*Total citations: 1757 (Google Scholar) *Total journal impact factors: 174.48 *H-index: 22 (as of 7/21/2016)

PROFESSIONAL ACTIVITIES

TEACHING

MentoringPostdoctoral fellows

2009-2011	Lamia Boric, M.D.	Physician, Utah Cancer Specialists
2011-2014	Tiffany Katz, Ph.D.	Postdoctoral Associate, Texas A&M University
2014-2016	Chunyu Cao, Ph.D.	Instructor, China Three Georges University
2016-	Ye Qin, M.D., Ph.D.	Postdoctoral Fellow, University of Pittsburgh
2016-	Hao Wu, M.D., Ph.D.	Visiting Fellow, University of Pittsburgh

Current positionUndergraduate Students

2010	Emily Platz, visiting student of Johns Hopkins University
2015-2017	Lin Chen, visiting student of Tsinghua University

High School Students

2013	Jennifer Han, UPCI Academy
2015	Jeewon Lee, UPCI Academy
2016	Kiera Regan, UPCI Academy

Ph.D. Candidacy Exam Committee

2013	Courtney Anderson, Molecular Pharmacology, University of Pittsburgh
2013	Kyle Knickelbein, Molecular Pharmacology, University of Pittsburgh
2015	Nolan Priedigkeit, Molecular Pharmacology, University of Pittsburgh
2015	Alison Nagle, Molecular Pharmacology, University of Pittsburgh
2016	Zachary Yochum, Molecular Pharmacology, University of Pittsburgh

Ph.D. Dissertation Committee

2013-present	Julia C. Woodcock, Pharmacology & Chemical Biology, University of Pittsburgh
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RESEARCH**1. Research Supports****Active Grant Support**

Project number	W81XWH-14-1-0237
Title:	Targeting histone abnormality in triple negative breast cancer
Role in project:	PI (Partnering PI: Davidson)
Year Inclusive:	8/1/2014-7/31/2017
Source:	Department of Defense – Breast Cancer Program Breakthrough
Amount:	\$ 565,048

Prior Grant Support

Project number	
Title:	Role of LSD2 in epigenetic gene silencing in breast cancer
Role in project:	PI
Year Inclusive:	2013-2014
Source:	UPCI Pilot Grant

Amount:	\$5,000
Project number	BCRF0016554
Title:	Role of histone demethylase in epigenetic regulation of gene expression in breast cancer
Role in project:	Co-I, PI: Davidson
Year Inclusive:	2014-2015
Source:	Breast Cancer Research Foundation
Amount:	\$200,000/yr (\$70,279 supports Dr. Huang's research)
Project number	
Title:	Targeting crosstalk between LSD1 and HDAC in triple negative breast cancer
Role in project:	PI
Year Inclusive:	2012-2013
Source:	UPMC Competitive Medical Research Fund
Amount:	\$25,000
Project number	P50 CA88843-08 (JHU PO#2009 12087) (Davidson)
Project number	BCRF0016554
Title:	Role of histone demethylase in epigenetic regulation of gene expression in breast cancer
Role in project:	Co-I, PI: Davidson
Year Inclusive:	2009-2014
Source:	Breast Cancer Research Foundation
Amount:	\$89,418/yr
Title:	Specialized Program in Research Excellence (SPORE in Breast Cancer)
Role in project:	Co-I, PI of Project 2-2
Year Inclusive:	2009-2012
Source:	NCI
Amount:	\$43,163/yr
Project number	
Title:	Crosstalk between histone demethylase and histone deacetylase: a novel epigenetic target for breast cancer
Role in project:	PI
Year Inclusive:	2011-2012
Source:	Samuel and Emma Winters Foundation
Annual direct cost:	\$9,000
Project number	DAMD 17-03-1-0376 (Huang)
Title:	Antineoplastic efficacy of novel polyamine analogues in human breast cancer
Role in project:	PI

Year Inclusive:	2003-2006
Source:	DOD Breast Cancer Research Program
Amount:	\$170,000
Project number	DAMD 17-00-1-0301 (Huang)
Title:	The role of histone deacetylase and DNA methylation in estrogen receptor expression in breast cancer
Role in project:	PI
Year Inclusive:	2002-2003
Source:	DOD Breast Cancer Research Program
Amount:	\$96,000

Pending Grant Support

NCI RO1	Targeting HDAC5-LSD1 axis in triple negative breast cancer
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2. Invited Seminars and Lectureships

2008.5.	AACR Special Conference-Cancer Epigenetics, Boston, MA, "Novel oligoamine analogues inhibit lysine specific demethylase 1 and activate silenced gene re-expression in colon cancer cells."
2009.6.	Polyamine Gordon conference, Waterville Valley, NH, "Histone Lysine-specific Demethylase (LSD1): an emerging epigenetic target for polyamine analogues in cancer therapy."
2009.9.	The 1 st UPCI Minisymposium on Biology, Treatment, and Prevention of Breast Cancer, Pittsburgh, PA, "Targeting epigenetic silencing in breast cancer: biology and translational implications".
2009.12.	Pittsburgh Chromatin Club Symposium, Pittsburgh, PA. "Targeting histone lysine specific demethylase 1 (LSD1) as a novel epigenetic strategy in cancer therapy".
2010.5.	University of Pittsburgh Cancer Institute Basic & Translational Research Seminar Series, Pittsburgh, PA. "Histone Lysine-specific Demethylase 1 (LSD1) as a potential therapeutic target for breast Cancer".
2011.9.	The 1 st Annual Retreat of Women's Cancer Research Center, University of Pittsburgh, "Epigenetics and breast cancer: exploding the box, or unraveling the chromatin"
2013.9.	The 24 th Annual Vascular Biology and Hypertension Symposium, University of Alabama at Birmingham, "Epigenetic regulation of estrogen receptor signaling in breast cancer: biology and therapeutic implications"
2015.10.	The 4 th International Breast Cancer Stem Cell Symposium, Suzhou, China, "Targeting epigenetic abnormality in breast cancer: biology and clinical implication"
2016.9.	The 2nd Great Lakes Breast Cancer Symposium, Pittsburgh, PA. "Targeting crosstalk between epigenetic modifiers in breast cancer"
2017.3.	University of Pittsburgh Cancer Institute Basic and Translational Research Seminar Series.

3. Invited Peer Review Activities

Journals: ♦ Amino Acids ♦ Breast Cancer Research ♦ Breast Cancer Research & Treatment ♦ BBA-Molecular Cell Research ♦ BMC Cancer ♦ Cancer Biology & Therapy ♦ Cancer Investigation ♦ Cancer Research ♦ Cancer Research Frontiers ♦ Carcinogenesis ♦ Cell Death and Differentiation ♦ Clinical Cancer Research ♦ Clinical & Experimental Metastasis ♦ Cancer Letters ♦ Cell Biochemistry and Biophysics ♦ Expert Opinion on Therapeutic Patents ♦ FEBS Letters (2016) ♦ Frontiers of Epigenomics ♦ Hormones and Cancer ♦ Journal of National Cancer Institute ♦ Life Science ♦ Medicinal Chemistry Communications ♦ Molecular and Cellular Endocrinology ♦ Molecular Carcinogenesis ♦ Neoplasia ♦ Oncotarget ♦ PLOS ONE ♦ Reproductive Biology and Endocrinology ♦ Scientific Report ♦ The Journal of Investigative Dermatology ♦ Translational Oncology

Book chapters: ♦ Handbook of Epigenetics, Second Edition, Elsevier

4. Editorial Boards

2011- Frontiers in Epigenomics
2013-2015 Cancer and Clinical Research

5. Study Section or Grant Review Services

Member of grant review panel

2010 US Army/DOD Breast Cancer Research Program
2015 US Army/DOD Breast Cancer Research Program

External reviewer for funding agency

2015 Israel Science Foundation
2016 UK Breast Cancer Now Research Fund
2016 UK Wellcome Trust Research Grant
2016 Canada Ontario Research Fund

SERVICE

University and Medical School Service

2000-01 President, International Association of Medical University of South Carolina
2013-2015 Poster judge, WCRC retreat, UPCI
2013 Poster judge, 24th Annual Vascular Biology and Hypertension Symposium, University of Alabama at Birmingham
2014 WCRC retreat planning committee
2014-2016 UPCI retreat judge
2015 UPCI retreat organization committee member
2015 UPCI Summer Academy, WCRC site director
2016 2nd Great Lakes Breast Cancer Symposium organizing committee

Community Service

2000-01 Vice President, Association of Chinese Scholar and students of Charleston SC
2015.11. Speaker, 2nd UPCI breast cancer advocacy boot camp

ORIGINAL ARTICLE

Functional interaction of histone deacetylase 5 (HDAC5) and lysine-specific demethylase 1 (LSD1) promotes breast cancer progression

C Cao^{1,2,3}, SN Vasilatos^{1,3}, R Bhargava^{1,4}, JL Fine^{1,4}, S Oesterreich^{1,2,3}, NE Davidson^{1,2,3} and Y Huang^{1,2,3}

We have previously demonstrated that crosstalk between lysine-specific demethylase 1 (LSD1) and histone deacetylases (HDACs) facilitates breast cancer proliferation. However, the underlying mechanisms are largely unknown. Here, we report that expression of HDAC5 and LSD1 proteins were positively correlated in human breast cancer cell lines and tissue specimens of primary breast tumors. Protein expression of HDAC5 and LSD1 was significantly increased in primary breast cancer specimens in comparison with matched-normal adjacent tissues. Using HDAC5 deletion mutants and co-immunoprecipitation studies, we showed that HDAC5 physically interacted with the LSD1 complex through its domain containing nuclear localization sequence and phosphorylation sites. Although the *in vitro* acetylation assays revealed that HDAC5 decreased LSD1 protein acetylation, small interfering RNA (siRNA)-mediated HDAC5 knockdown did not alter the acetylation level of LSD1 in MDA-MB-231 cells. Overexpression of HDAC5 stabilized LSD1 protein and decreased the nuclear level of H3K4me1/me2 in MDA-MB-231 cells, whereas loss of HDAC5 by siRNA diminished LSD1 protein stability and demethylation activity. We further demonstrated that HDAC5 promoted the protein stability of USP28, a bona fide deubiquitinase of LSD1. Overexpression of USP28 largely reversed HDAC5-KD-induced LSD1 protein degradation, suggesting a role of HDAC5 as a positive regulator of LSD1 through upregulation of USP28 protein. Depletion of HDAC5 by shRNA hindered cellular proliferation, induced G1 cell cycle arrest, and attenuated migration and colony formation of breast cancer cells. A rescue study showed that increased growth of MDA-MB-231 cells by HDAC5 overexpression was reversed by concurrent LSD1 depletion, indicating that tumor-promoting activity of HDAC5 is an LSD1 dependent function. Moreover, overexpression of HDAC5 accelerated cellular proliferation and promoted acridine mutagen ICR191-induced transformation of MCF10A cells. Taken together, these results suggest that HDAC5 is critical in regulating LSD1 protein stability through post-translational modification, and the HDAC5–LSD1 axis has an important role in promoting breast cancer development and progression.

Oncogene advance online publication, 23 May 2016; doi:10.1038/nc.2016.186

INTRODUCTION

Lysine-specific demethylase 1 (LSD1) is the first identified FAD-dependent histone demethylase that has been typically found in association with a transcriptional repressor complex that includes CoREST, HDAC1/2, BHC80 and others.^{1–4} A role for elevated expression of LSD1 has been implicated in tumorigenesis in various cancers including breast cancer.^{3,5–9} Studies from our and other laboratories consistently showed that inhibition of LSD1 hindered proliferation of breast cancer cells.^{6,8,10} Lim *et al.*⁶ reported that LSD1 is highly expressed in estrogen receptor-negative breast cancers. A recent study found that LSD1 is significantly overexpressed in high-grade ductal carcinoma *in situ* or invasive ductal carcinoma versus low/intermediate ductal carcinoma *in situ*.¹¹ These studies point to a tumor-promoting role for LSD1 in breast cancer. We were among the first to report the use of small-molecule compounds and preclinical treatment strategies that have promise to work through this target in cancer.^{8,9,12} The development of novel LSD1 inhibitors is progressing rapidly. For example, a new generation of (bis)urea/(bis)thiourea LSD1 inhibitors displayed improved potency against LSD1 in cancer cells.¹³ A newly reported

GSK-LSD1 inhibitor exhibited interesting cell type-specific inhibition against small-cell lung cancer cells in preclinical models.¹⁴

However, how LSD1 is upregulated in breast cancer and the precise role of LSD1 in breast cancer development are still unclear. Our most recent work showed that small interfering RNA (siRNA)-mediated inhibition of HDAC5 led to a significant increase of H3K4me2, a known substrate of LSD1, suggesting a potential role of HDAC5 in regulating LSD1 activity.¹⁰ However, little is known about the precise role of HDAC5 and mechanisms underlying its regulation on LSD1 activity in breast cancer. HDAC5 is an important member of class IIa histone deacetylase (HDAC) isozymes with important functions in transcriptional regulation, cell proliferation, cell cycle progression and cellular developmental activities.^{15,16} HDAC5 has been shown to have important roles in many diseases including cancer.^{17,18} In this study, we addressed the following clinically relevant issues that have been understudied: (1) Is elevation of LSD1 expression associated with HDAC5 overexpression during breast cancer development? (2) How is LSD1 regulated by HDAC5 in breast cancer? (3) What is the role of the HDAC5–LSD1 axis in breast cancer initiation, proliferation and metastasis? To answer these questions, we delineated the

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mechanisms underlying the functional link between LSD1 and HDAC5 in chromatin remodeling and demonstrated that these two important chromatin modifiers closely cooperate to mediate proliferation, cell cycle and metastasis of breast cancer cells.

RESULTS

HDAC5 and LSD1 proteins are coordinately expressed in human breast cancer

To study the potential association of HDAC5 and LSD1 in breast cancer, we first examined mRNA levels of HDAC5 and LSD1 in human immortalized normal mammary epithelial MCF10A cells, fully malignant MCF10A-CA1a cells transformed from MCF10A cells with transfection of *HRA5*,¹⁹ and several human breast cancer cell lines. Quantitative PCR (qPCR) studies showed that there was no clear association of mRNA expression between HDAC5 and LSD1 in breast cancer cell lines (Figure 1a). The Oncomine-TCGA database showed moderate change of the mRNA level of LSD1 and HDAC5 in IBC (Supplementary Figures 1a and b). mRNA levels of both HDAC5 and LSD1 are altered in ~6% of breast cancer patients (www.cbioportal.org) without an apparent association with specific subtypes (Supplementary Figures 1c and d). However, protein expression of both HDAC5 and LSD1 was significantly elevated in malignant breast cell lines compared with MCF10A (Figure 1b), and protein levels of HDAC5 and LSD1 were positively correlated (Figure 1c). The correlation of HDAC5 and LSD1 protein expression was further validated in 50 primary breast cancers using immunohistochemical staining with validated antibodies (Supplementary Figures 2a and b). The χ^2 analysis showed a statistically significant correlation between HDAC5 and LSD1 protein expression in these tumors (Figure 1d). Furthermore, the immunohistochemistry (IHC) analysis showed that breast cancer tissues ($n=18$) expressed significantly higher level of HDAC5 and LSD1 than matched-normal adjacent tissues ($n=18$) (Figure 1e). The mean H-score for HDAC5 staining in stage 3 breast tumors ($n=25$) was statistically significantly higher than stage 2 counterparts ($n=25$). The mean H-score of LSD1 staining for stage 3 tumors was also higher than that of stage 2 tumors with a P -value of 0.07 (Figure 1f). These results were further validated with independent manual H-score evaluations by two breast cancer pathologists with moderate interobserver concordance (Supplementary Figures 3a and b). Taken together, these findings suggest that HDAC5 and LSD1 proteins are coordinately over-expressed in breast cancer cell lines and tissue specimens.

Physical interaction of LSD1 and HDAC5 in breast cancer cells

To address whether LSD1 and HDAC5 physically interact, a co-immunoprecipitation study was carried out in MDA-MB-231 and MCF10A-CA1a cells transiently transfected with pcDNA3.1 or pcDNA3.1-FLAG-HDAC5 plasmids. After immunoprecipitation (IP) with LSD1 antibody, we found that both endogenous and exogenous HDAC5 proteins were co-immunoprecipitated with LSD1 protein (Figure 2a). The interaction between native LSD1 and HDAC5 was further validated in additional breast cancer cell lines (Figure 2b). A similar result was obtained in the reciprocal immunoprecipitation using anti-FLAG antibody to confirm that

LSD1 was co-immunoprecipitated with FLAG-HDAC5 (Figure 2c). To precisely map the HDAC5 domain(s) responsible for interaction with LSD1, we expressed a series of HDAC5 deletion mutants engineered in pcDNA3.1-FLAG plasmids in MDA-MB-231 cells (Figure 2d). Immunoprecipitation assays of cells transfected with full-length HDAC5 complementary DNA (cDNA) confirmed the HDAC5–LSD1 interaction and deletion of an N-terminal myocyte enhancer factor-2 (MEF2) binding domain (HDAC5- Δ 1) alone had no impact on HDAC5–LSD1 interaction. However, removal of both the MEF2 domain and nuclear localization sequence (NLS) (HDAC5- Δ 2) completely abolished HDAC5–LSD1 interaction. Further deletion of an N-terminal HDAC and nuclear export sequence (HDAC5- Δ 3) and MEF2 domain (HDAC5- Δ 4) did not adversely alter LSD1 binding with HDAC5 fragments (Figure 2e). Immunofluorescence studies showed nuclear localization of full-length HDAC5, HDAC5- Δ 1, HDAC5- Δ 3 and HDAC5- Δ 4. Depletion of the NLS-containing domain (HDAC5- Δ 2) completely blocked HDAC5 nuclear translocation (Figure 2f). *In vitro* pull-down assays by using His-tag recombinant LSD1 protein incubating with HDAC5 full-length or deletion mutants validated that HDAC5 domain containing NLS element is essential for interaction with LSD1 (Supplementary Figure 4).

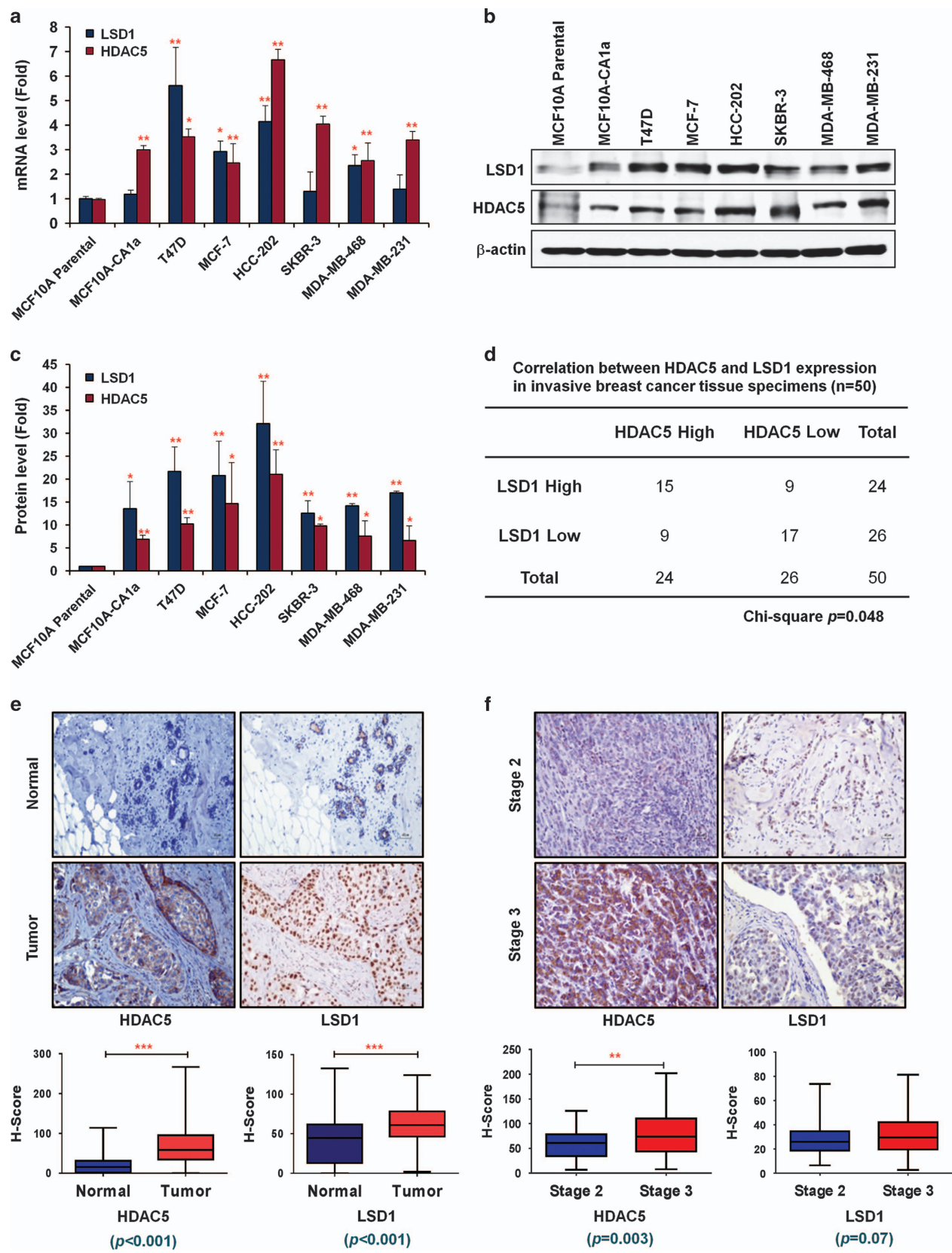
HDAC5 promotes LSD1 protein stability and activity

Next, we examined whether the mRNA or protein levels of HDAC5 and LSD1 were affected by their interaction with each other. Overexpression of HDAC5 in MDA-MB-231 cells failed to alter LSD1 mRNA expression, but led to a significant increase of LSD1 protein expression (Figures 3a and b). HDAC5 knockdown by siRNA attenuated LSD1 protein expression without affecting its mRNA level (Figures 3c and d). The effect of LSD1 on HDAC5 expression was subsequently assessed using our previously established MDA-MB-231-LSD1-KD cells.¹⁰ Depletion of LSD1 exerted no effect on HDAC5 mRNA or protein levels (Figures 3e and f). Simultaneous overexpression of pcDNA3.1-HDAC5 with HDAC5 siRNA significantly reversed the decrease of LSD1 (Supplementary Figure 5a). These results suggest that HDAC5 functions as an upstream regulator that governs LSD1 protein stability via post-translational regulation. Quantitative immunoblots showed that levels of H3K4me1/2 and AcH3K9, the substrates for LSD1 and HDAC5, respectively, were downregulated by HDAC5 overexpression, whereas loss of HDAC5 exerted the opposite effect (Figure 3g; Supplementary Figure 5b), suggesting a critical role of HDAC5 in governing chromatin modifying activity of LSD1. The cycloheximide chase assay showed that overexpression of HDAC5 significantly extended LSD1 protein half-life, whereas depletion of HDAC5 by siRNA decreased LSD1 protein half-life in MDA-MB-231 cells (Figures 3h and i; Supplementary Figure 5c). To determine whether other recognized LSD1 cofactors or HDACs exert similar effects on LSD1 protein stability, MDA-MB-231 cells were treated with siRNA against several LSD1 complex cofactors (CoREST, HDAC1 and HDAC2) or other class II HDAC isozymes (HDAC 4, 6, 7, 9, 10), respectively. Transfection with siRNA probes effectively knocked down mRNA expression of target genes without affecting LSD1 protein level (Figure 3j; Supplementary Figure 6a). To confirm the qPCR results, quantitative immunoblotting (IB) was performed and showed depletion of

Figure 1. Correlated overexpression of HDAC5 and LSD1 protein in breast cancer. **(a)** The levels of mRNA expression of HDAC5 and LSD1 in breast cancer cell lines versus MCF10A cells (set as fold 1) using real-time qPCR with β -actin as an internal control. **(b)** Immunoblots with anti-HDAC5 and LSD1 antibodies in indicated cell lines. β -actin protein was blotted as a loading control. **(c)** Histograms represent the mean protein levels of HDAC5 or LSD1 in three determinations relative to β -actin \pm s.d. as determined by quantitative immunoblots. **(d)** 50 primary human invasive breast tumor samples were immunostained with antibodies against HDAC5 or LSD1. The χ^2 study was performed by using median H-scores as the cutoff for high- versus low-protein expression. **(e)** Representative HDAC5 and LSD1 staining (200 \times) in invasive breast carcinoma and adjacent normal tissue specimens from one representative patient. H-scores represent average staining intensity in breast tumors ($n=18$) versus adjacent normal breast tissue ($n=18$). **(f)** Representative HDAC5 and LSD1 staining (200 \times) in stage 2 and 3 invasive breast carcinoma specimens. H-scores represent average staining intensity in stage 3 breast tumors ($n=25$) versus stage 2 breast tumors ($n=25$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t -test.

CoREST led to insignificant change of LSD1 protein stability (Supplementary Figure 6b and 6c). Together, these results strengthen the conclusion that HDAC5 functions as a positive regulator of LSD1 protein in breast cancer cells.

HDAC5 regulates LSD1 protein stability through modulation of the LSD1-associated ubiquitination system
Protein ubiquitination assays indicated that HDAC5 overexpression significantly attenuated LSD1 polyubiquitination (Figure 4a),



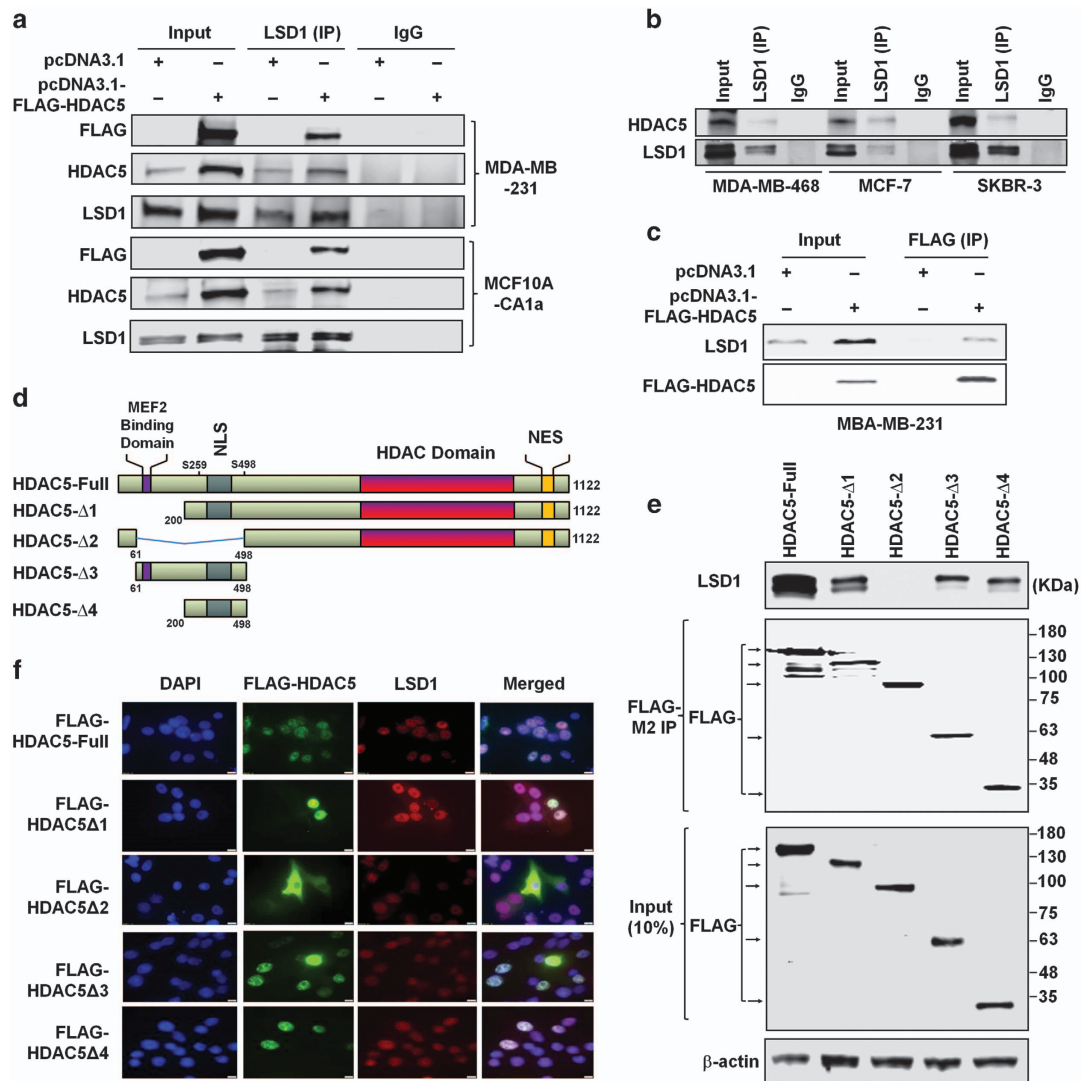


Figure 2. HDAC5 and LSD1 physically interact in breast cancer cells. **(a)** MDA-MB-231 or MCF10A-CA1a cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids. IP was performed with anti-LSD1 antibody followed by immunoblotting (IB) with anti-LSD1, anti-FLAG or anti-HDAC5 antibodies, respectively. **(b)** Whole-cell lysates were immunoprecipitated with anti-LSD1 antibody followed by IB with anti-LSD1, anti-FLAG or anti-HDAC5 antibodies, respectively. **(c)** MDA-MB-231 cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5-FLAG plasmids, and IP was performed with anti-FLAG followed by IB with anti-LSD1 and anti-FLAG antibodies, respectively. **(d)** Schematic representation of full-length and deletion mutants of HDAC5-FLAG constructs. **(e)** FLAG-tagged full-length or deletion mutants of HDAC5 were expressed in MDA-MB-231 cells. Extracts were immunoprecipitated with anti-FLAG antibody, and bound LSD1 was examined by IB using anti-LSD1 antibody. IB with anti-FLAG was used to detect the levels of FLAG-tagged HDAC5 full-length or deletion mutants in IP and input (10%) samples. **(f)** MDA-MB-231 cells were transfected with plasmids expressing FLAG-tagged full-length or deletion mutants of HDAC5 proteins. Immunofluorescence study was performed using anti-FLAG antibody. 4,6-Diamidino-2-phenylindole was used as a control for nuclear staining. All the experiments were performed three times with similar results.

whereas depletion of HDAC5 by siRNA facilitated LSD1 polyubiquitination (Supplementary Figure 7a). Recently, Jade-2 and USP28 were identified as specific E3 ubiquitin ligase and deubiquitinase for LSD1, respectively.^{20,21} Our study showing that increase of LSD1 protein expression by Jade-2 siRNA and decrease of LSD1 protein expression by USP28 siRNA in MDA-MB-231 cells confirmed the roles of Jade-2/USP28 as LSD1 ubiquitin ligase/deubiquitinase in breast cancer cells (Figure 4b; Supplementary Figure 7b). qPCR studies demonstrated that mRNA level of either Jade-2 or USP28 was not altered by HDAC5 knockdown or overexpression (Figure 4c). The regulation of HDAC5 on protein expression of Jade-2 or USP28 was subsequently assessed. Due to the lack of highly specific antibody against Jade-2, plasmids expressing Jade-2-FLAG fusion protein were transfected into cells as an alternative approach. MDA-MB-231 and MCF10A-CA1a cells

expressing Jade-2-FLAG protein were simultaneously treated with HDAC5 siRNA to evaluate the effect of HDAC5 on Jade-2 protein expression. Immunoblot showed that depletion of HDAC5 did not change the protein level of Jade-2 (Figure 4d). However, overexpression of HDAC5 led to significant increase of USP28 protein expression in both cell lines (Figure 4e). *In vitro* pull-down assay using His-tag recombinant LSD1 protein incubated with USP28-FLAG protein indicated a direct interaction of LSD1 and USP28 (Supplementary Figure 4), and HDAC5 overexpression significantly attenuated USP28 polyubiquitination (Supplementary Figure 7c). To understand whether HDAC5 may stabilize LSD1 protein through upregulation of USP28 protein stability, a rescue study was carried out in MDA-MB-231 and MCF10A-CA1a cells using concurrent transfection of HDAC5 siRNA and USP28 expression plasmids, and showed that overexpression of USP28

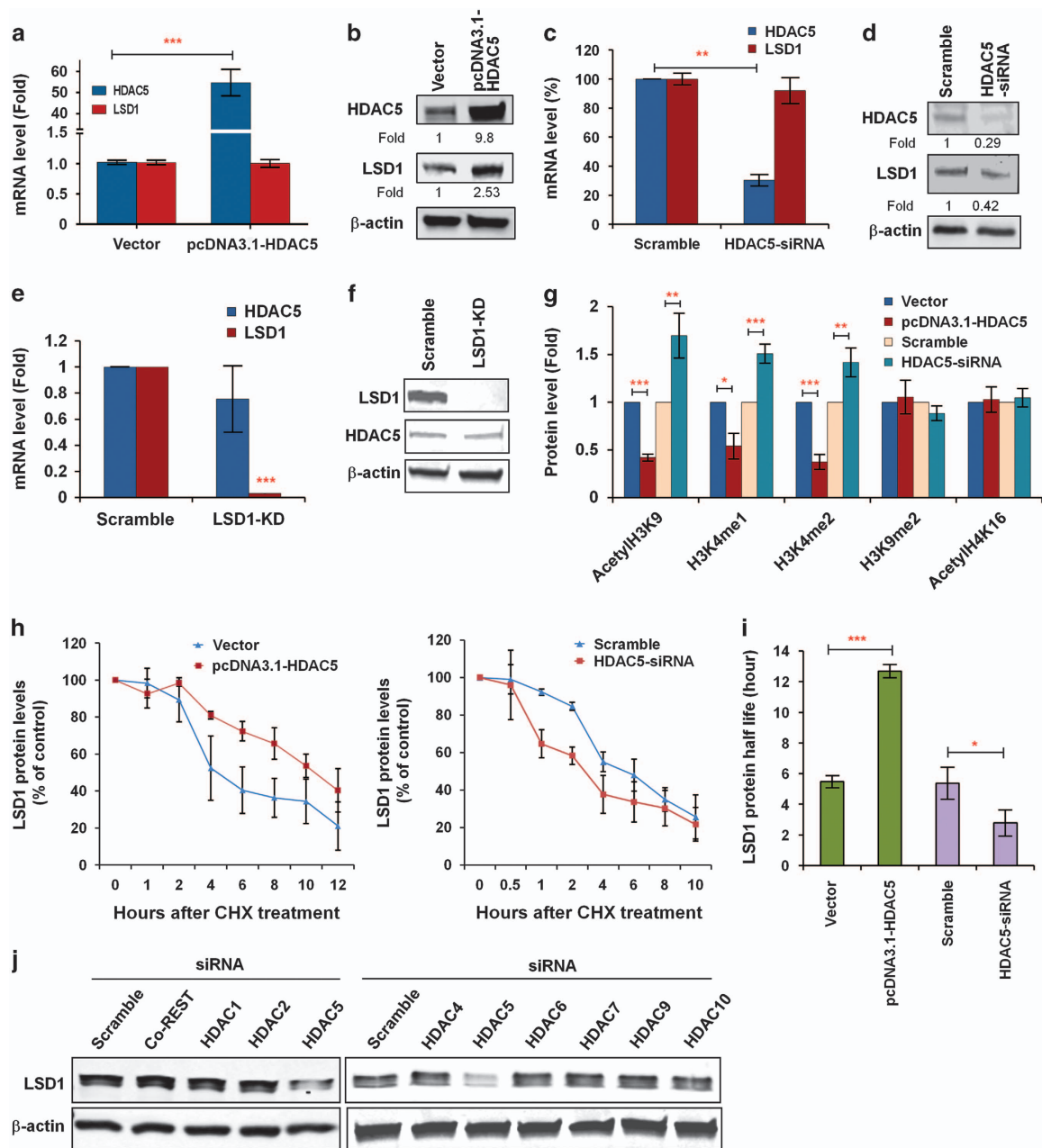


Figure 3. HDAC5 stabilizes LSD1 protein in breast cancer cells. **(a)** MDA-MB-231 cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 for 48 h. mRNA expression of HDAC5 and LSD1 was measured by quantitative real-time PCR with β -actin as an internal control. **(b)** MDA-MB-231 cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids for 48 h. Effect of HDAC5 overexpression on LSD1 protein expression in MDA-MB-231 cells was evaluated by immunoblots with anti-LSD1 and anti-HDAC5 antibodies. **(c)** MDA-MB-231 cells were transfected with scramble siRNA or HDAC5 siRNA for 48 h. Effect of HDAC5 knockdown on LSD1 mRNA expression was examined by quantitative real-time PCR with β -actin as internal control. **(d)** Effect of HDAC5 siRNA on LSD1 protein expression in MDA-MB-231 cells. **(e)** Effect of depletion of LSD1 on mRNA expression of HDAC5 in MDA-MB-231-Scramble or MDA-MB-231-LSD1-KD cells. **(f)** Effect of LSD1-KD on protein expression of HDAC5 in MDA-MB-231-scramble or MDA-MB-231-LSD1-KD cells. **(g)** MDA-MB-231 cells were transfected with control vector pcDNA3.1, pcDNA3.1-HDAC5, scramble siRNA or HDAC5 siRNA for 48 h and analyzed by immunoblots for nuclear expression of indicated histone marks. Proliferating cell nuclear antigen was used as loading control. **(h)** Effect of HDAC5 overexpression or siRNA on LSD1 protein half-life in cycloheximide chase study. **(i)** Measurement of LSD1 half-life using the CalcuSyn program. **(j)** Effect of siRNA knockdown of LSD1 cofactors or class II HDACs on LSD1 protein level. All the experiments were performed three times. Bars represent the mean of three independent experiments \pm s.d. * P < 0.05, ** P < 0.01, *** P < 0.001, Student's t -test.

completely blocked the destabilization of LSD1 by HDAC5 depletion (Figure 4f; Supplementary Figure 7d). In an additional rescue experiment, overexpression of HDAC5 failed to promote LSD1 protein expression when cells were simultaneously treated with USP28 by siRNA (Supplementary Figure 7e). All these data

support the notion that HDAC5 stabilizes LSD1 protein by enhancing protein expression of its deubiquitinase.

To examine whether interaction of HDAC5 with the LSD1/USP28 complex deacetylates LSD1 or USP28, *in vitro* protein acetylation assays was first carried out by incubating GST-tagged recombinant

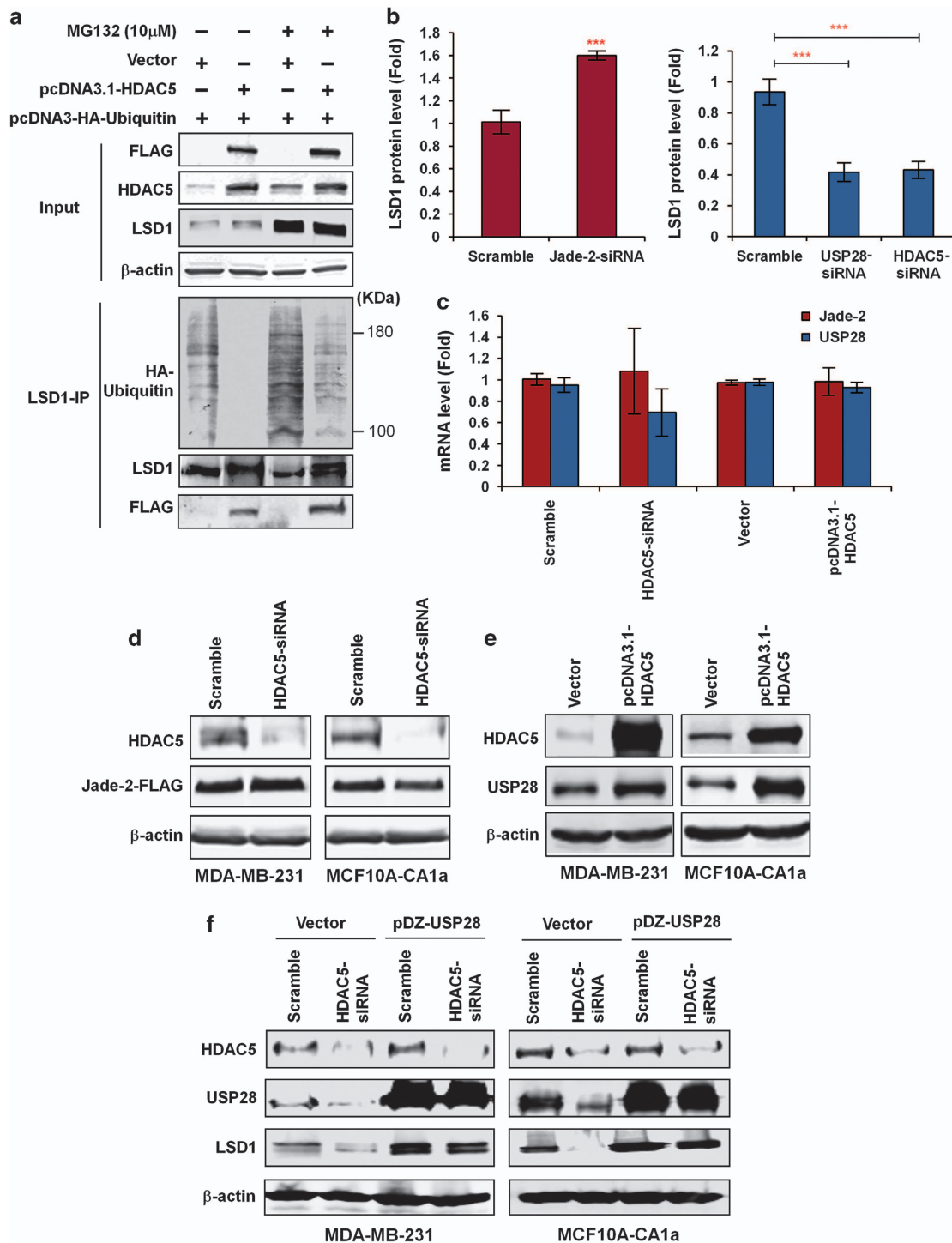


Figure 4. HDAC5 regulates LSD1 by altering USP28 stability. **(a)** MDA-MB-231 cells transfected with pcDNA3.1-FLAG, pcDNA3.1-FLAG-HDAC5 or pcDNA3-HA-ubiquitin plasmids were treated with or without proteasome inhibitor 10 μ M MG132 for 10 h followed by IP using LSD1 antibody and immunoblots with anti-HA, LSD1 or HDAC5 antibodies. **(b)** Effect of siRNA of Jade-2, USP28 and HDAC5 on LSD1 protein expression in MDA-MB-231 cells. Results represent the mean of three independent experiments \pm s.d. *** P < 0.001, Student's *t*-test. **(c)** MDA-MB-231 cells were transfected with scramble siRNA, HDAC5 siRNA, control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids for 48 h. mRNA expression of Jade-2 and USP28 was measured by qPCR. β -actin was used as an internal control. **(d)** MDA-MB-231 or MCF10A-CA1a cells were simultaneously transfected with pcDNA3.1-FLAG-Jade-2 and HDAC5 siRNA for 48 h and subjected to immunoblots with anti-HDAC5 or Jade-2 antibodies. β -actin was used as loading control to normalize target protein levels. **(e)** After MDA-MB-231 or MCF10A-CA1a cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 plasmids for 48 h, IB was performed for expression of HDAC5 and USP28. **(f)** MDA-MB-231 or MCF10A-CA1a cells were transfected with scramble or HDAC5 siRNA alone, or in combination with pDZ-USP28 for 48 h. Whole-cell lysates were analyzed for protein levels of HDAC5, USP28 and LSD1. β -actin was used as loading control to normalize target protein levels. The experiments were performed three times with similar results.

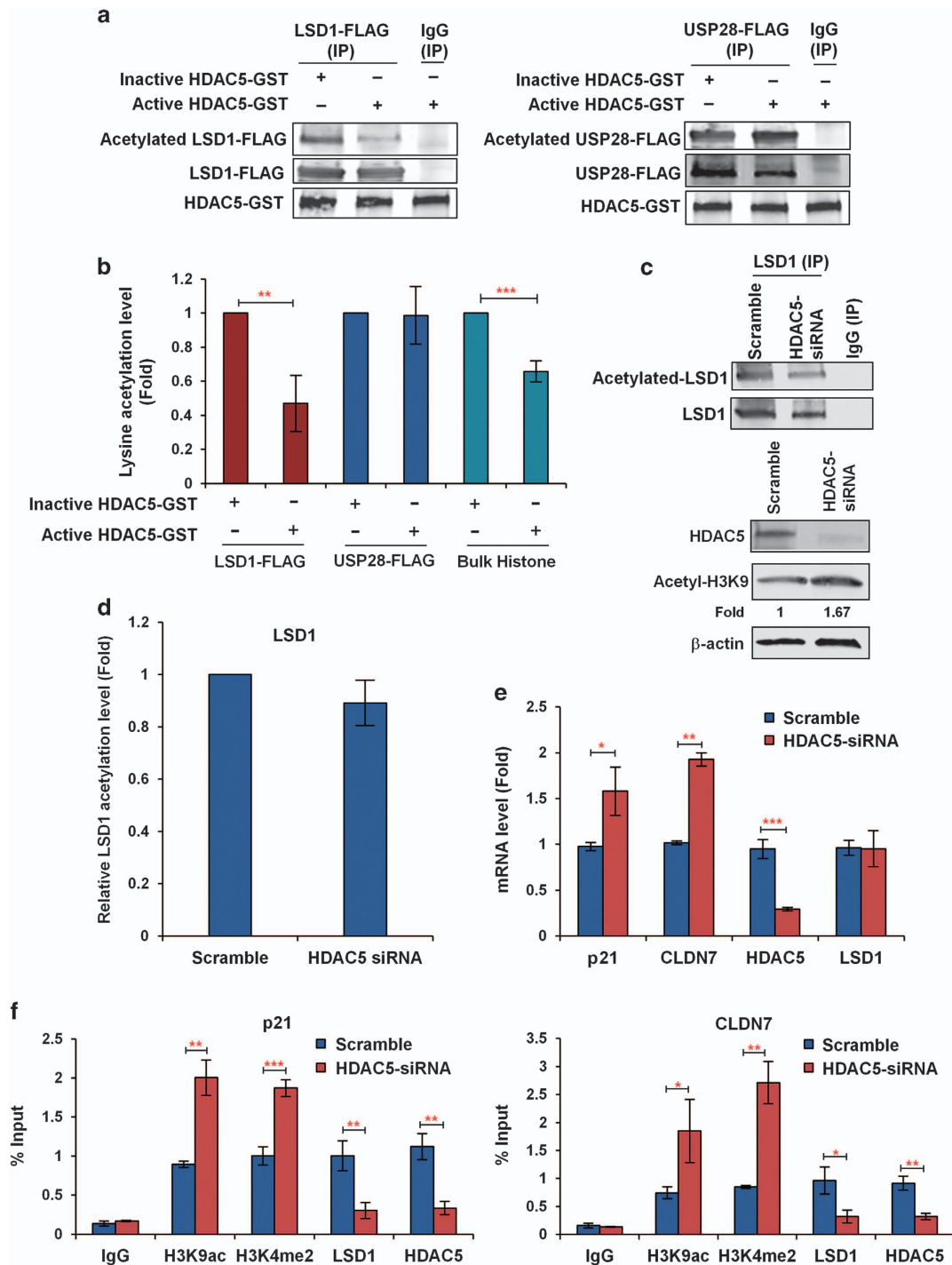
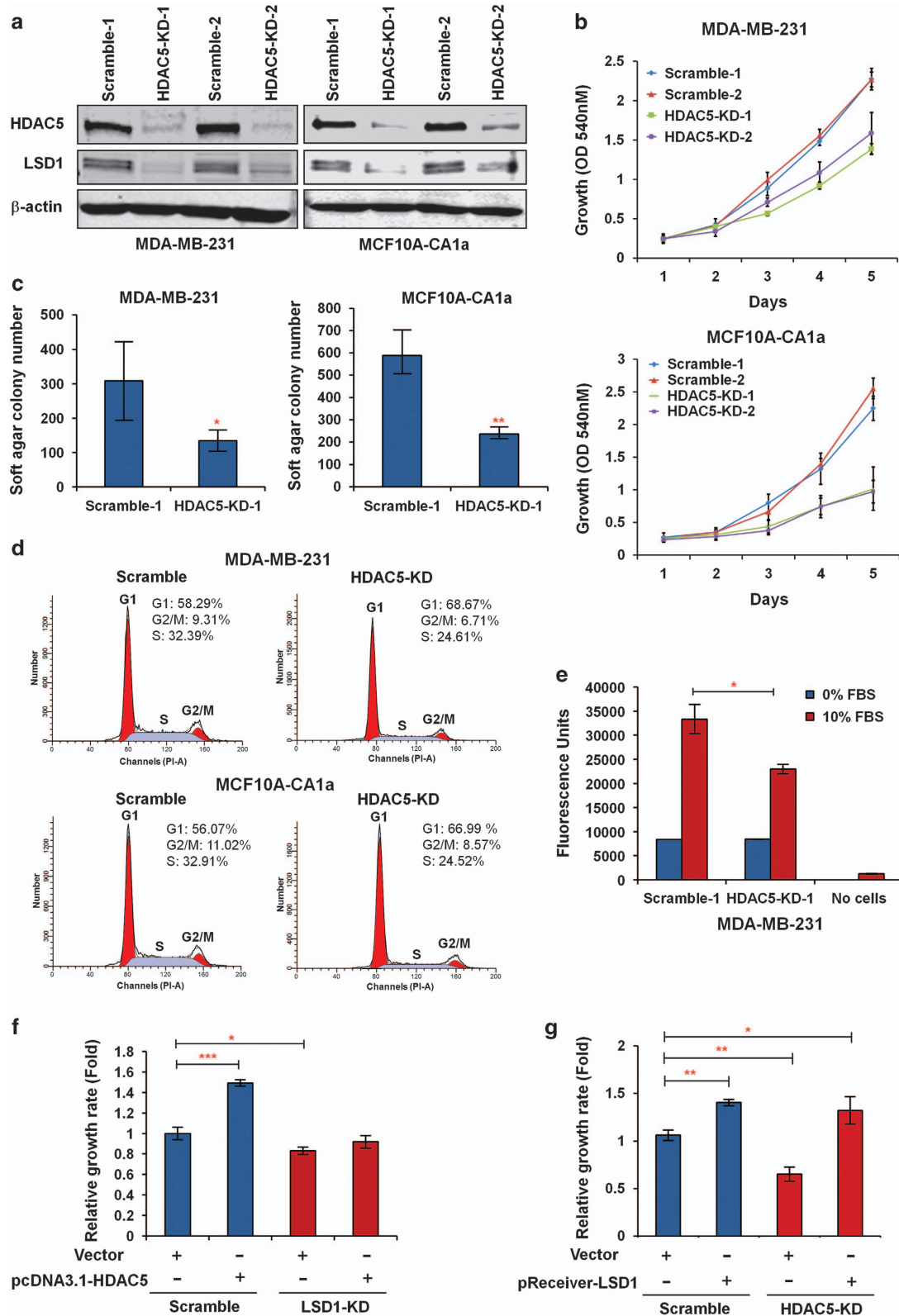


Figure 5. Effect of HDAC5 on protein acetylation of LSD1/USP28 and transcription of LSD1 target genes. **(a)** The immunoprecipitates of FLAG using FLAG-M2 agarose from MDA-MB-231 cells overexpressing FLAG-tagged USP28 or FLAG-tagged LSD1 were used as substrates for protein deacetylation assay. IgG was used as negative control. Active or heat inactivated recombinant human GST-tagged HDAC5 protein were mixed with immunoprecipitates and incubated at 37 °C for 6 h as described in 'Materials and Methods'. The reactions were then subjected to immunoblots with anti-acetyl lysine antibody. FLAG-tagged USP28 or LSD1 proteins were probed with anti-FLAG antibody. HDAC5-GST protein was probed with anti-HDAC5 antibody. **(b)** Histograms represent the means of levels of acetyl-LSD1, acetyl-USP28 and acetyl-histone determined by quantitative IB using infrared IB detection and analysis. **(c)** MDA-MB-231 cell transfected with scramble or HDAC5 siRNAs for 48 h. LSD1 or IgG antibodies were added to cell lysate. IP was performed with anti-LSD1 antibody followed by IB with anti-acetyl lysine and anti-LSD1 antibodies, respectively. Effect of HDAC5 siRNA on AcetylH3K9 protein expression in MDA-MB-231 cells was examined by IB with anti-acetyl-H3K9 antibody. **(d)** Histograms represent the means of relative levels of acetyl-LSD1 determined by quantitative IB using infrared IB detection and analysis. **(e)** mRNA expression of indicated genes in MDA-MB-231 cells transfected with scramble siRNA or HDAC5 siRNA. Data are means \pm s.d. of three independent experiments. **(f)** Quantitative chromatin immunoprecipitation (ChIP) analysis was used to determine the occupancy by acetyl-H3K9, H3K4me2, LSD1 and HDAC5 at promoters of p21 or CLDN7 in MDA-MB-231 cells transfected with scramble or HDAC5 siRNA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

HDAC5 protein with cellular pull-down of LSD1-FLAG or USP28-FLAG by IP, and immunoprecipitates of IgG was incubated with recombinant HDAC5 protein as negative control of assays (Figure 5a). Bulk histone was used as control substrate (Supplementary Figure 8). Quantitative immunoblots using antibody against pan-acetylated lysine showed that HDAC5 reduced

acetylation level of LSD1 without altering the acetylation status of USP28 (Figures 5a and b). Next, the *in vivo* effect of HDAC5 depletion on LSD1 acetylation was investigated in MDA-MB-231 cells transfected with scramble or HDAC5 siRNAs. After immunoprecipitation with LSD1 antibody or IgG (negative control), IB was performed and the results showed that expression levels of both



total LSD1 protein and acetylated LSD1 protein were decreased by HDAC5 depletion (Figure 5c). Quantitative immunoblots indicated that the relative acetylation level of LSD1 was not statistically altered by HDAC5 siRNA in MDA-MB-231 cells (Figure 5d). Acetyl-H3K9 was used as control of substrate and its expression was increased by HDAC5 siRNA (Figure 5c). These results suggest that inhibition of HDAC5 alone is not sufficient enough to increase LSD1 acetylation in breast cancer cells.

Inhibition of HDAC5 reactivates expression of LSD1 target genes. In cancer cells, amplified LSD1 expression is frequently associated with abnormal suppression of key tumor suppressor genes.^{3,22} We next examined whether expression of LSD1 target tumor suppressor genes could be reactivated following HDAC5 inhibition. Loss of expression of cyclin-dependent kinase inhibitor p21 and epithelial marker claudin-7 (CLDN7) has been reported to be associated with an aggressive phenotype of breast cancer.^{23,24} The transcription activity of p21 and CLDN7 has been found to be suppressed by enhanced activity of LSD1 in breast cancer.^{6,25} Transfection of HDAC5 siRNA resulted in significantly increased mRNA expression of p21 and CLDN7 in MDA-MB-231 cells (Figure 5e). Quantitative chromatin immunoprecipitation assays revealed that depletion of HDAC5 decreased occupancy of both HDAC5 and LSD1, and increased enrichment of H3K4me2 and acetyl-H3K9 at the promoters of both genes (Figure 5f). These data suggest that transcriptional de-repression of these genes lies largely in the cooperation between HDAC5 and LSD1 at key active histone marks.

Inhibition of HDAC5–LSD1 axis hinders breast cancer proliferation and invasion

To explore the functional role of the HDAC5–LSD1 axis in regulating breast cancer development, stable knockdown of HDAC5 mRNA (HDAC5-KD) was generated in MDA-MB-231 and MCF10A-CA1a cells by infection with short hairpin RNA (shRNA) lentiviral particles. Similar to the effect of transient inhibition of HDAC5 by siRNA, stable knockdown of HDAC5 expression significantly reduced LSD1 protein expression in two independent HDAC5-KD clones (Figure 6a). Loss of HDAC5 in both clones hindered cell proliferation and colony formation in soft agar (Figures 6b and c). The flow cytometry analysis showed that inhibition of HDAC5 resulted in a greater fraction of cells accumulated at G1 phase and reduction of the S-phase cell fraction (Figure 6d; Supplementary Figure 9). Moreover, loss of HDAC5 attenuated motility and invasion of MDA-MB-231 cells in a Boyden chamber assay (Figure 6e). A rescue experiment indicated that HDAC5 overexpression promoted growth of MDA-MB-231-Scramble cells, but failed to alter the growth of MDA-MB-231-LSD1-KD cells (Figure 6f). An additional rescue study revealed that LSD1 overexpression rescued growth inhibition by HDAC5 depletion in MDA-MB-231-HDAC5-KD cells (Figure 6g). Taken together, these results demonstrate that tumor-promoting activity of HDAC5 is dependent on LSD1 activity in breast cancer cells.

Overexpression of HDAC5 promotes mutagen-induced tumorigenic development in MCF10A cells

To address whether enhanced interaction between HDAC5 and LSD1 is a critical epigenetic alteration driving tumorigenic transformation of breast cancer, we generated two MCF10A cell lines overexpressing HDAC5 (MCF10A-HDAC5). Stable overexpression of HDAC5 in MCF10A cells increased LSD1 protein level and promoted cell proliferation of both clones (Figures 7a and b), indicating a growth-promoting role for HDAC5 in MCF10A cells. Inhibition of LSD1 by shRNA significantly hindered MCF10A growth and reversed the growth promotion mediated by HDAC5 overexpression, suggesting that HDAC5 promotes MCF10A growth in an LSD1 dependent manner (Figure 7c; Supplementary Figure 10). To evaluate if MCF10A-HDAC5 cells have altered susceptibility to tumorigenesis, MCF10A-Vector and MCF10A-HDAC5 cells were cultured for 7 months in medium containing 500 ng/ml ICR191. ICR191 generates genomic instability and genetic variability, and has been successfully used to induce epithelial cell transformation in several models including MCF10A.^{26,27} MCF10A-HDAC5 cells were subsequently tested for the capacity of anchorage-independent growth in soft agar for 4 weeks. The soft agar colony formation study demonstrated that ICR191 treatment improved the ability of MCF10A cells to form growing colonies, and overexpression of HDAC5 significantly promoted ICR191-induced colony formation in MCF10A cells (Figure 7d). To determine the role of LSD1 in HDAC5 enhanced tumorigenic transformation induced by ICR191, scramble control and LSD1 shRNA lentivirus particles were infected into MCF10A-Vector or MCF10A-HDAC5 cells, which had been treated with ICR191 for 7 months, and the soft agar growth assays showed that loss of LSD1 in MCF10A-HDAC5 cells significantly abolished cellular ability in colony formation (Figure 7e). A model illustrating the role of HDAC5–LSD1 axis in breast cancer development is proposed based on the above findings (Figure 7f).

DISCUSSION

High levels of HDAC5 have been found to be associated with poor survival in multiple cancer types.^{28,29} LSD1 overexpression has been reported to be a poor prognostic factor in basal-like breast cancer, a subtype with aggressive clinical characteristics.^{6,30} In this study, the IHC analysis showed that breast cancers expressed higher levels of HDAC5 compared to the matched-normal adjacent breast tissue. Importantly, our study found a positive correlation between HDAC5 and LSD1 proteins in breast tumor cell lines and patient tissue specimens. Increased expression of HDAC5 and LSD1 is correlated with higher stage of breast cancer in our exploratory study. These findings suggest that the coordinated overexpression of HDAC5 and LSD1 may serve as potential novel prognostic markers as well as possible therapeutic targets for breast cancer. More robust studies will be necessary to understand the precise role of elevated protein expression levels of HDAC5 and LSD1 in the risk stratification of breast cancer patients.

Figure 6. HDAC5–LSD1 axis is implicated in breast cancer progression. **(a)** Depletion of HDAC5 by shRNA lentivirus infection downregulated LSD1 protein expression in MDA-MB-231 and MCF10A-CA1a cells. **(b)** Scramble shRNA and HDAC5-KD cells were analyzed for growth and viability by crystal violet assays. **(c)** Soft agar colony formation for HDAC5-KD and scramble control of MDA-MB-231 and MCF10A-CA1a cells. **(d)** Scramble shRNA and HDAC5-KD cells were harvested and stained for DNA with propidium iodide for the flow cytometric analysis. The fractions corresponding to G1, S and G2/M phases of the cell cycle are indicated. **(e)** The Boyden Chamber transwell migration assays for cell invasion for MDA-MB-231-Scramble or MDA-MB-231-HDAC5-KD-1 cells. **(f)** MDA-MB-231-Scramble or MDA-MB-231-LSD1-KD cells were transfected with control vector pcDNA3.1 or pcDNA3.1-HDAC5 for 5 days and crystal violet assays for growth were carried out. **(g)** MDA-MB-231-Scramble or MDA-MB-231-HDAC5-KD cells were transfected with empty or pReceiver-LSD1 expression plasmids for 5 days and crystal violet assays for growth were carried out. Bars represent the means of three independent experiments \pm s.d. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

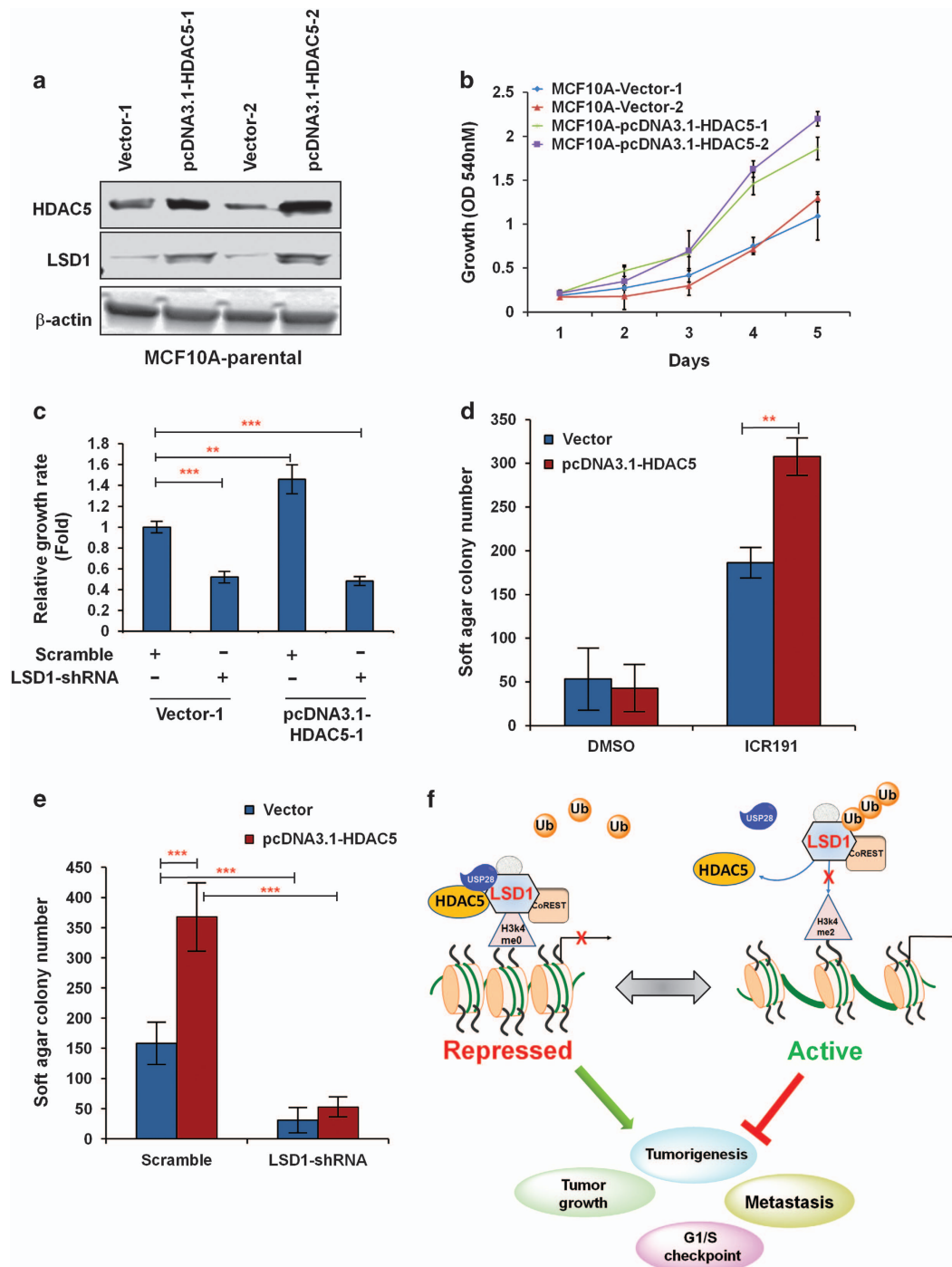


Figure 7. Effect of HDAC5 on growth and mutagen-induced tumorigenic transformation in MCF10A cells. **(a)** pcDNA3.1 or pcDNA3.1-HDAC5 transfected MCF10A cells (clone 1 and 2) were analyzed for protein levels of HDAC5 and LSD1 by immunoblots with anti-HDAC5 and anti-LSD1 antibodies. **(b)** The crystal violet assay for growth of MCF10A stably transfected with control vector or pcDNA3.1-HDAC5 plasmids. **(c)** MCF10A-Vector-1 or MCF10A-HDAC5-1 cells were infected with scramble or LSD1 shRNA lentivirus particles for 5 days followed by crystal violet assays for growth. **(d)** MCF10A cells transfected with pcDNA3.1 or pcDNA3.1-HDAC5 plasmids were treated with dimethyl sulfoxide or 500 ng/ml ICR191 for 7 months followed by soft agar colony formation assays. **(e)** After treatment with 500 ng/ml ICR191 for 7 months, MCF10A-HDAC5 cells were infected with scramble control or LSD1 shRNA lentivirus particles and soft agar colony formation assay was carried out. **(f)** Proposed model of the role of HDAC5-LSD1 axis in breast cancer development. Bars represent the means of three independent experiments \pm s.d. ** $P < 0.01$, *** $P < 0.001$, Student's *t*-test.

LSD1 protein stability is controlled by several post-translational modifications such as ubiquitination and methylation.^{20,21,31} However, the precise mechanism of how LSD1 protein stability is regulated is still not understood. A previous study reported that stable depletion of CoREST facilitated LSD1 degradation in HeLa

cells.³² However, siRNA-mediated knockdown of CoREST alone in breast cancer cells failed to destabilize LSD1 protein, suggesting additional layers of control of LSD1 protein stability are required in breast cancer. In this study, we observed for the first time that LSD1 protein stability is promoted by HDAC5. We further found

that the HDAC5 domain containing NLS is essential for LSD1–HDAC5 interaction. The NLS element provides docking sites for 14–3–3 chaperone binding and has been shown to be critical for HDAC5 import into the nucleus and the regulation of its repressor activity.^{17,33} Although an *in vitro* assay demonstrated that HDAC5 reduced LSD1 acetylation, HDAC5 siRNA treatment in breast cancer cells failed to alter acetylation of LSD1 protein. Our *in vivo* results suggest that LSD1 acetylation is likely regulated by a large complex that may involve additional protein deacetylases or cofactors. Further studies are needed to identify the regulatory complex and clarify the precise role of HDAC5 in regulation of LSD1 acetylation in breast cancer cells.

Our studies revealed that HDAC5 regulates LSD1 via enhancement of the protein stability of deubiquitinase USP28. High expression of USP28 has been found to promote the progression of breast and colon cancers.^{20,34} Importantly, USP28 has been reported to deubiquitinate important tumor growth regulators such as c-Myc and TP53BP1 that are involved in MYC proto-oncogene stability and DNA damage response checkpoint regulation, respectively.^{35,36} Our pilot microarray study revealed that inhibition of the HDAC5–LSD1 axis down-regulates c-Myc expression (data not shown). Sen *et al.*³⁷ recently reported that HDAC5 is a key component in the temporal regulation of p53-mediated transactivation. All of these findings imply an interaction of HDAC5/LSD1 axis and USP28-associated ubiquitin–proteasome system in regulating downstream targets involved in tumor development. USP28 has been well-characterized for its role in promoting tumorigenesis, and thus is a potential candidate target in cancer therapy. Given the current inability to use drugs to directly target USP28-driven cancer proliferation, our study suggests a novel alternative approach of targeting USP28 stability by development of HDAC5-specific inhibitors in cancer.

Our findings provide supportive evidence showing that HDAC5 control of cell proliferation is largely dependent on LSD1 stabilization. Furthermore, in this study, we showed that non-transformed MCF10A cells overexpressing HDAC5 significantly promoted ICR191-induced transformation of MCF10A cells. The overexpressed HDAC5 is consistently associated with upregulated LSD1 protein expression over the entire course of transformation induction. These data indicate that enhanced crosstalk between HDAC5 and LSD1 may represent a critical mechanism contributing to breast tumorigenesis. HDAC inhibitors hold great promise for cancer therapy. Despite the promising clinical results produced by the HDAC inhibitors in treatment of hematological cancers such as T-cell lymphoma, no apparent clinical evidence indicates that HDAC inhibitors work effectively as a monotherapy against solid tumors including breast tumors.^{38–41} From a clinical perspective, our novel findings have significance for design and development of novel combination strategies targeting HDAC5–LSD1 axis as an alternative approach for improvement of therapeutic efficacy of HDAC inhibitors in breast cancer.

As summarized in Figure 7f, we show for the first time that LSD1 protein stability is promoted by HDAC5 through the LSD1 associated ubiquitin–proteasome system, confirming that the regulation of LSD1 by HDAC5 is a post-translational event. Our novel findings also provide supportive evidence that an orchestrated interaction between HDAC5 and LSD1 is a critical epigenetic mechanism to suppress transcriptional activities of important tumor suppressor genes that may contribute to breast cancer development.

MATERIALS AND METHODS

Reagents and cell culture conditions

MDA-MB-231, MDA-MB-468, MCF-7, T47D, HCC-202 and SK-BR-3 cell lines were obtained from the ATCC/NCI Breast Cancer SPORC program. MCF10A-parental and MCF10A-CA1a cells were gifts from Dr Saraswati Sukumar

(Johns Hopkins University). Cells were cultured in growth medium as described previously.^{10,42}

Tissue microarrays and immunohistochemistry

Tissue microarrays (US Biomax, Rockville, MD, USA) were stained using LSD1 or HDAC5 antibodies. Standard staining procedure for paraffin sections was used for IHC according to manufacturer's recommendations (Vector Labs Inc., Burlingame, CA, USA). Monoclonal antibodies were used for detection of LSD1 (1:800; Cell Signaling, Danvers, MA, USA) and HDAC5 (1:100; Santa Cruz, CA, USA). The staining was visualized using diaminobenzidine, and quantitated using IHC Profiler, an ImageJ plugin (National Institutes of Health, Bethesda, MD, USA).⁴³ H-scores were calculated as previously described.⁴⁴ The manual scoring of H-scores was also carried out by two breast cancer pathologists.

Plasmid construction and stable transfection

Plasmids pcDNA3.1(+)-FLAG, pcDNA3.1(+)-FLAG-HDAC5 and pDZ-FLAG-USP28 were purchased from Addgene (Cambridge, MA, USA). pReceiver-FLAG-LSD1 was obtained from Gene Copoeia (Rockville, MD, USA). A FLAG-tagged ORF cDNA clone for Jade-2 was purchased from GenScript (Piscataway, NJ, USA). pcDNA3-HA-ubiquitin was obtained from Dr Yong Wan (University of Pittsburgh). HDAC5 deletion mutants were engineered into pcDNA3.1(+)-FLAG-HDAC5 by PCR with primers shown in Table S1. HDAC5-Δ2 was constructed by digesting full-length plasmids with SacII from amino acid 61 to 489. Stable transfection was carried out using Lipofectamine 3000 transfection reagent (Life Technologies, Grand Island, NY, USA), and colonies were selected with 800 μg/ml G418.

siRNA and shRNA treatment and stable cell line generation

Pre-designed siRNA and non-targeting scramble siRNA (Santa Cruz) were transfected into cells following the manufacturer's protocol. Cells were collected 48 h post-transfection for further analysis. Scramble control, LSD1-specific or HDAC5-specific shRNA lentiviral particles (Santa Cruz) were infected into cells according to manufacturer's protocol. Cells were treated with 10 μg/ml puromycin 72 h after infection. Single colonies were analyzed for expression of LSD1 or HDAC5 via immunoblots.

RNA extraction and qPCR

Total RNA extraction and cDNA synthesis used the methods described previously.¹⁰ Quantitative real-time PCR was performed on the StepOne real-time PCR system (Life Technologies). All of the TaqMan gene expression assays were pre-designed and obtained from Life Technologies.

Western blotting

Western blotting was performed as previously described.^{12,45,46} Antibodies used in this study were shown in Supplementary Table S2. Membranes were scanned with Li-Cor BioScience Odyssey Infrared Imaging System (Lincoln, NE, USA).

Crystal violet and cell invasion assays

The crystal violet proliferation assays were performed as described in our previous study.⁴⁷ The invasive capability of breast carcinoma cells was tested with Millipore QCM 24-well invasion assay kit (Merck KGaA, Germany) according to manufacturer's protocol.

Soft agar colony formation assay

A total of 1.2% Bacto-agar (BD Biosciences, Franklin Lakes, NJ, USA) was autoclaved and mixed with growth medium to produce 0.6% agar. The mixture was quickly plated and solidified for 45 min. Cells were suspended in 0.6 ml 2× growth medium and mixed gently with 0.6 ml 0.8% agar/medium. Overall 1 ml of cells with 0.4% agar/medium mixture was added onto plate for solidification. Colony formation was examined using stereo microscopy and analyzed (CellSens Dimension, Olympus, Shinjuku, Tokyo, Japan).

Flow cytometry analysis

Cells were collected and fixed with 70% ethanol. The cell pellet was then treated with 1% TritonX-100. Cells were subsequently resuspended in 50 μg/ml propidium iodide (Sigma, St Louis, MO, USA) containing RNaseI (Roche, Indianapolis, IN, USA) followed by analysis on the LSR II XW4400 workstation (BD Biosciences).

Immunofluorescence

After 48 h of transfection, cells were fixed with 4% paraformaldehyde and incubated with primary antibodies (1:250) overnight at 4 °C. After washing, cells were incubated with fluorescence-labeled secondary antibody (1:100). After washing, coverslips were placed on a glass slide using UltraCruz mounting medium (Santa Cruz) before fluorescence microscope examination.

Immunoprecipitation, ubiquitination and protein half-life assays

The cell lysate was obtained by using immunoprecipitation lysis buffer as described previously.⁴⁸ LSD1 or IgG antibodies were added to cell lysate. Protein G-plus agarose beads (Santa Cruz) or Flag-M2 affinity gel were collected and subjected to IB. HA-Ubiquitin, pcDNA3.1-Flag-HDAC5 or empty vector plasmids were co-transfected into cells for 38 h. Cells were then treated with 10 μ M MG132 for 10 h and collected for immunoprecipitation assay with protein G-plus agarose beads. For half-life studies 48 h after transfection with pcDNA3.1-HDAC5 or HDAC5 siRNA, cells were treated with 100 μ g/ml cycloheximide and then collected at indicated times for IB.

Protein acetylation assay

The immunoprecipitates of FLAG-M2 agarose from MDA-MB-231 cells overexpressing FLAG-tag USP28 or FLAG-tag LSD1 were used as substrates for the protein deacetylation assay. Pull-down of IgG was used as negative control. A total of 0.25 μ g of recombinant human GST-tagged HDAC5 protein (Creative BioMart, NY, NY) was mixed with 30 μ l immunoprecipitates or 1.5 μ g bulk histone at 37 °C for 6 h in a buffer containing 40 mM Tris–HCl (pH 8.0), 2.5 mM MgCl₂, 50 mM NaCl, 2 mM KCl, 0.5 mM DTT, 1 mM EDTA and protease inhibitor. The reactions were then subjected to immunoblots with anti-acetyl lysine antibody (EMD Millipore, Billerica, MA, USA). FLAG-tagged USP28 or LSD1 and bulk histone were probed with anti-FLAG antibody or H3 antibody as loading control. Inactive HDAC5–GST protein was used as negative control by heating recombinant protein at 95 °C for 5 min. *In vivo* protein acetylation assay was performed using cell lysate of MDA-MB-231 cell transfected with scramble and HDAC5 siRNAs. LSD1 or IgG antibodies were added to cell lysate. Protein G-plus agarose beads (Santa Cruz) were collected and subjected to IB with anti-acetyl lysine or LSD1 antibodies.

Chromatin immunoprecipitation

Chromatin immunoprecipitation assay was performed as described previously.¹² Primary antibodies against HDAC5, LSD1, H3K4me2 and acetyl-H3K9 were used as indicated for immunoprecipitation of the protein–DNA complexes. PCR primer sets used for amplification of precipitated fragments were shown in Supplementary Table S1. Input DNA was used for normalization.

Statistical analysis

Data were represented as the mean \pm s.d of three independent experiments. The quantitative variables were analyzed by the two-tailed Student's *t*-test. The χ^2 study was used to assess the correlation between HDAC5 and LSD1 protein expression by using median H-scores as the cutoff for high- versus low-protein expression. *P*-value < 0.05 was considered statistically significant for all tests. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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